REMARKS

Applicants gratefully acknowledge the courtesy shown by Examiner Li and Supervisory Examiner Crouch in the interview with inventor Nils Lonberg and Applicants' representative Paul Fehlner on September 21, 2004. The interview addressed all of the grounds for rejection, including (1) enablement of the human chromosomal fragment SC20 and consequently claim 1 as amended; (2) enablement of transgenic mice in which the endogenous heavy and light chain genes may not be knocked out; (3) deposit of genetic constructs that enable generation of the claimed mice; (4) unexpected results of transchromosomal-transgenic mice, as shown by the exemplary data in the specification, which rules out obviousness; (5) and agreement that U.S. Patent No. 6,632,976 does not anticipate the pending claims, nor is there an issue of non-statutory double patenting with respect to the claims of the '976 patent and the pending claims.

Reconsideration of the present application in view of the above amendments and following remarks is respectfully requested. Claims 1, 2, 6-9, and 11 were pending. As set forth above, Applicants have hereby cancelled claim 2 without prejudice to the filing of any divisional, continuation, or continuation-in-part application thereon, or directed toward the subject matter of the claims as originally filed or as subsequently amended. In addition, Applicants submit that claim 1 has been hereby amended to more clearly define the subject matter encompassed by the Applicants' invention. Support for the amendment to claim 1 is found in the application as originally filed, in part, at page 29, lines 5-22, and original claim 2. No new matter has been added. Therefore, claims 1, 6-9, and 11 are currently pending.

REJECTION UNDER 35 U.S.C. §112, FIRST PARAGRAPH (ENABLEMENT)

In the Office Action dated March 26, 2004, claims 1, 2, 6-9 and 11 were rejected under 35 U.S.C. §112, first paragraph, for lack of enablement. In particular, it is alleged that making transgenic mice with chromosomal transfer is unpredictable and, therefore, undue experimentation would be required to make a transgenic mouse comprising a transchromosome carrying any human heavy chain locus or any *IgH* locus from Chromosome 14. Furthermore, it is

alleged that the specification only teaches mice having null endogenous Ig loci and, therefore, it is allegedly uncertain whether mice having endogenous Ig loci could make sufficient levels of human antibodies. In addition, it is alleged that mice having endogenous Ig loci would make chimeric antibodies and the specification allegedly fails to teach a use for chimeric antibodies. Finally, it is noted that a hSC(20)/KCo5 mouse with null endogenous Ig heavy and light chain loci may not be readily available or obtainable by the methods described in the specification and that a deposit of a mouse embryo would be enabling.

1. Enablement of human chromosome fragments

Applicants respectfully traverse this ground of rejection and submit that the disclosure of the instant specification is commensurate in scope with the claims and that no undue experimentation is required to practice the invention. The present invention is directed, in pertinent part, to a transgenic mouse comprising two human immunoglobulin loci, wherein one of the human immunoglobulin loci is a human heavy chain locus carried by transchromosome SC20, and the other locus is a human light chain locus carried by a transgene integrated into the genome of the mouse. As an initial matter, Applicants respectfully submit that the instant specification teaches what transchromosomes are (*see, e.g.*, page 10, lines 7-13; page 38, lines 17-30), how to make transchromosomes generally (*see, e.g.*, page 39, lines 2-5) or with deposited material (*see, e.g.*, page 29, lines 5-22), and how to make transgenic animals having transchromosomes (*see, e.g.*, page 39, line 24-27). Furthermore, various transchromosomes have been successfully used to make transgenic animals (*see, e.g.*, Tomizuka *et al.*, *Nature Genetics 16*:133, 1997; Tomizuka *et al.*, *Proc. Nat'l. Acad. Sci. USA 97*:722, 2000, and Hernandez *et al.*, *Hum. Mol. Genet. 8*:923, 1999). Notwithstanding enablement of claim 1 prior to the present amendment, to expedite prosecution of the instant application and without acquiescing to the instant rejection, Applicants have hereby amended claim 1 to specifically recite transchromosome SC20. Thus, the instant claims satisfy the enablement requirements of 35 U.S.C. §112, first paragraph as the Examiner stated in the Office Action (*see* pages 2-3) and as agreed during the interview.

2. Enablement of mice with active endogenous Ig loci

With regard to the requirement of mice having null endogenous Ig loci, Applicants respectfully disagree with the rejection. As pointed out in the interview, at the time of the invention one of skill in the art would not have required null endogenous Ig loci in a transgenic mouse to use the mouse; indeed, usually the endogenous lambda light chain is not null. Furthermore, mice containing transchromosome and transgene constructs in a non-Ig null background can be bred to Ig-null mice and, consequently, are useful in this respect as well. Such mice may be preferred once generated, but they are not essential to successfully practice the invention.

Dr. Lonberg pointed out in the interview that the claimed animals are capable of producing a starting material that is not easily obtained: high affinity human sequence antibodies against a human antigen. This high affinity results from selection and affinity maturation of the human sequence V regions, particularly the V(D)J regions, of the antibody. The human constant region in such antibodies is a convenient surrogate for development of high affinity secondary antibody responses, but not essential.

Furthermore, while in some cases an antibody from a transgenic animal might be used without further modification, the invention also contemplates extracting just the V region recombinant gene segment from the antibody. This V region might be joined with a different C region for altered functionality, or used for further modification (*see* the instant Specification at page 42, lines 13-27). The Specification (at page 40, lines 8-12) describes obtaining V regions directly from B cells in transgenic mice or from hybridomas generated from such B cells. Paragraph 137 of the Specification (as filed, not as published) describes using the V region segments in phage display techniques. Partial antibody sequences can be used to express intact antibodies (*see* Specification, paragraph 141). Human antibodies (as well as chimeric and humanized antibodies) are typically produced using recombinant expression in suitable host cells, such as CHO cells (*see* Specification, paragraphs 153-156), rather than by growing up hybridomas. Thus, whether endogenous antibodies are present in the transgenic animal is irrelevant because the technology exists, and has existed, to obtain the human sequence antibodies generated in these same mice, and to make and use these antibodies using routine technology.

Applicants respectfully submit that when transgenic technology was first developing, animals with intact endogenous antibody gene loci were successfully used to generate human antibodies (*see, e.g.*, Taylor *et al.*, *Nuc. Acids Res.* 1992, 20:6287-95; "Taylor"). Taylor describes successfully generating fully human sequence antibodies in transgenic mice containing intact endogenous Ig loci. B cells expressing both human mu and human kappa Ig proteins were identified, albeit at low level (about 0.5% of the total B cell population). Even at this relatively low level, 29 independent light chain immunoglobulin and 49 independent heavy chain genes were sequenced, and thus available to incorporate in any immunoglobulin construct (*see* Taylor, Figures 3 and 4, which report the CDR3 [VDJ] sequences of human heavy and light chains). Thus, as of 1992, detectable levels of human antibodies could be generated and obtained from transgenic mice with intact endogenous Ig loci.

A later paper demonstrated the ability to sequence V regions from antibody chimeras (*see, e.g.*, Taylor *et al.*, *Int. Immunol.* 1994, 6:579, Figure 7). This establishes that as of 1994, the ability to extract V regions from chimeric antibodies and the genes encoding them would not have proved a barrier to enablement of the claimed transgenic animals, even if they did produce chimeric antibodies.

As discussed at the interview, the presence of chimeric antibodies would not differ whether the mouse endogenous Ig loci were null or not. Indeed, with respect to chimeric antibodies, *i.e.*, antibodies that have a human V region and murine C region, the status of the endogenous Ig loci has no impact. The Ig null mice still have switch and C region heavy chain genes. Thus, as pointed out in the specification, chromosomal translocation and transwitching events occur even in mice that are null for the endogenous Ig loci (*see* the Specification at page 63, lines 1-6). Since chimeric antibodies are possible whether or not the mouse has an endogenous Ig-null background, this reason for the rejection should be withdrawn.

Similarly, even in Ig null backgrounds, hybrid human/mouse antibodies can form (*see* the Specification at page 62, lines 31-32; see also Tomizuka *et al.*, *PNAS* 2000, 97:722-27, of record, Figure 5). Again, this result does not render the invention inoperative or non-functional. Indeed, as explained above, some of the first mice expressing human sequence immunoglobulins

did so despite endogenous murine immunoglobulin genes, and the presence of hybrid antibodies was not an impediment to selecting fully human sequence antibodies or the nucleic acids encoding such antibodies.

Furthermore, animals having endogenous Ig loci, while carrying a transchromosome and transgene according to the instant invention, can be used for breeding with animals that have inactivated or suppressed endogenous Ig loci to arrive at animals such as those encompassed by claims 6 and 7. The Specification exemplifies breeding to combine genetic characteristics in Example 1 (breeding CMD heavy chain knockout heterozygous animals to JHD knockout animals to generate CMD/JHD heterozygous animals homozygous for endogenous heavy chain inactivation); Example 2 (breeding KCo5 transgenic mice with human heavy chain transgenic, endogenous immunoglobulin locus mutant, mice); and Example 3 (breeding hCF(SC20) mice with the endogenous murine Ig locus inactivated to KCo5 mice, which also had the endogenous murine Ig locus inactivated). Furthermore, the '976 patent specifically describes, as one method of generating null endogenous Ig loci mice, "... in which a progeny retaining a human chromosome is obtained from a human chromosome-transferred chimeric mouse, followed by mating said progeny with a mouse in a strain deficient in a mouse antibody gene" (col. 21, lines 15-19). Example 73 of the '976 patent describes generating a mouse strain with transchromosomes for human heavy and light chain genes and inactivation of the endogenous Ig loci by repeated mating of transchromosomal mice with endogenous Ig null mice (col. 92, lines 54-67). Thus, the Specification and the level of skill in the art establish enablement of mice containing intact endogenous Ig loci along with a transchromosome and a transgene as intermediates in a breeding program with mice having inactivated Ig loci.

3. Enablement of DNA constructs

With respect to deposits, Applicants cannot determine whether the suggestion of depositing specific mouse embryos for enablement purposes is an actual rejection. Regardless, Applicants respectfully submit that transchromosome and transgene deposits are described in the instant specification (*see, e.g.*, at page 29, line 5 through page 30, line 2). As is known in the art

and described in the instant specification, transgenic animals according to the instant invention can be made using the described deposits, which satisfies the enablement requirement. Applicants submit that nothing more than objective enablement is required in order to meet the requirements of 35 U.S.C. §112, first paragraph. In particular, as stated by the Court of Customs and Patent Appeals:

> As a matter of Patent Office practice, then, a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented *must* be taken as in compliance with the enabling requirement of the first paragraph of § 112 *unless* there is reason to doubt the objective truth of the statements contained therein which must relied on for enabling support. *In re Marzzochi* 169 U.S.P.Q. 367, 369 (C.C.P.A. 1971) (emphasis in original)

In the instant case, Applicants have provided detailed enablement of the recited claims including, for example, methods for making transchromosomes (*see, e.g.*, page 38, line 17 through page 39, line 23) and transgenes (*see, e.g.*, page 33, lines 3-25), and methods for making transgenic animals of the currently claimed invention (*see, e.g.*, page 39, line 24 through page 40, line 6; and Example 3). The Examiner relies on an unsupported passage in Green *et al.* (US 2003/0093820), which is contrary to the art (U.S. Patent No. 6,632,976, Tomizuka *et al.*) and the teachings of the instant specification. Furthermore, there is an issued U.S. patent directed to the methods of making the transchromosomal animals (U.S. Patent No. 6,632,976). Consequently, there is a presumption that the technology is enabled. Transgenic mice, whether containing a minilocus or gene fragement, were well known in the art (*see* the Specification at page 2, lines 1-9).

Accordingly, Applicants respectfully submit that the grounds for the rejection under 35 U.S.C. §112, first paragraph has been overcome and, therefore, request that it be withdrawn.

REJECTION UNDER 35 U.S.C. §112, SECOND PARAGRAPH

In the Office Action, claim 2 was rejected under 35 U.S.C. §112, second paragraph, as indefinite. In particular, it is alleged that "the transchromosome SC20" lacks antecedent basis.

Applicants respectfully submit that this ground of rejection has been rendered moot because claim 2 has been hereby cancelled without prejudice. Accordingly, Applicants respectfully request that this rejection be withdrawn.

REJECTION UNDER 35 U.S.C. §§102(e) AND 102(f)

In the Office Action, claims 1, 2, 6-9 and 11 were rejected under 35 U.S.C. §102(e) and 102(f) as anticipated by U.S. Patent No. 6,632,976 (Tomizuka *et al.*). More specifically, it is alleged that Tomizuka *et al.* teach a transgenic mouse having a transchromosome, with the endogenous Ig gene disrupted, and a YAC vector that could be used to introduce a foreign gene, according to the instant invention.

Applicants respectfully traverse this ground of rejection and submit that the cited reference fails to meet every limitation of the instant claims and, therefore, Tomizuka *et al.* fail to anticipate the claimed invention. As discussed during the interview, Tomizuka *et al.* does not teach or suggest a mouse comprising two human immunoglobulin loci, wherein one of the human immunoglobulin loci is a human heavy chain locus carried by transchromosome SC20, and the other locus is a human light chain locus carried by a transgene integrated into the genome of the mouse. Tomizuka *et al.*, exclusively teaches the use of transchromosome technology (*see* col. 20, lines 45-66; *see* also Examples 18, 19, 20, 35, 36, 37, 3846, 4756, 5762, 63, 64, 65, 66, 70, 71, 72, and 73), do not teach the use of transchromosome technology in combination with transgene technology.

Tomizuka *et al.* teach a mouse wherein mouse cells retain a foreign chromosome or fragment thereof that is maintained independent of the mouse chromosomes and the foreign chromosome or fragment thereof comprises a human antibody gene. Tomizuka *et al.* pertains to transchromosome technology, which addresses deficiencies in the transgenic art, such as limits on the size of DNA capable of being transferred into mouse cells. Contrary to the assertion in the

Office Action, Tomizuka *et al.* are silent as to the use of transchromosomes and transgenes in combination, much less the combination in a single mouse as provided by the instant invention. Rather, Tomizuka *et al.* discuss the deficiencies of using a YAC vector for inserting a foreign gene and only in the background of the invention (*i.e.*, in the discussion of prior art; *see* Tomizuka *et al.*, column 2, lines 15-36). To be anticipating, the identical invention must be shown in as complete detail as is contained in the claim (*Richardson v. Suzuki Motor Co.*, 9 USPQ2d 1913, 1920, Fed. Cir. 1989) and the elements must be arranged as required by the claim (*In re Bond*, 15 USPQ2d 1566, Fed. Cir. 1990).

Accordingly, Applicants respectfully submit that the instant invention distinguishes patentably over Tomizuka *et al.* under the provisions of 35 U.S.C. §102(e) and 102(f). The Examiner agreed with this conclusion at the interview (*see* the Examiner Interview Summary Record). Applicants, therefore, request that this rejection be withdrawn.

REJECTION UNDER 35 U.S.C. §103(a)

In the Office Action, claims 1, 2, 6-9 and 11 were rejected under 35 U.S.C. §103(a) as unpatentable over U.S. Patent No. 5,770,429 (Lonberg *et al.*) by U.S. Patent No. 6,632,976 (Tomizuka *et al.*). More specifically, it is alleged that it would have been obvious for a person having ordinary skill in the art at the time the invention was made to breed mice as taught by Lonberg *et al.* and Tomizuka *et al.* to arrive at the instant invention.

As discussed at the interview, creating a mouse that contained a transchromosome for expressing the human heavy chain genes and a transgene for the human light chain genes yielded an unexpected result: a mouse with much greater efficiency in generating antigen-specific, antibody-secreting B cells than either the transchromosomal prior art of Tomizuko or the transgenic prior art of Lonberg, taken individually, additively, or on average. In the instant case, the recovery of a larger number of hybridomas from each fusion, as compared to Tomizuka *et al.* or Lonberg *et al.* mice, was unexpected (*see, e.g.*, specification at page 28, lines 31-33). In addition, the transgenic animals of the instant invention are unexpectedly superior at generating antigen-specific human monoclonal antibodies (*see, e.g.*, specification Example 5, at page 65, lines 7-8; Example 6,

at page 66, lines 7-9; and Example 7, at page 67, lines 5-8). As discussed at the interview, and agreed to by the Examiners, a person having ordinary skill in the art confronted with the same problems as the instant inventors and with no knowledge of the claimed invention would not have expected the combination of a transchromosome for the heavy chain and a transgene for the human light chain to have worked so well.[1] *See, e.g., In re Corkill*, 226 U.S.P.Q. 1005, Fed Cir. 1985; *In re Chupp*, 2 U.S.P.Q.2d 1437, Fed Cir. 1987.

The data in the specification establish these unexpected results for a number of different antigens, including human CD4 (page 65), human G-CSF (page 66), and human serum albumin (page 67, paragraph 205). Thus, the demonstration of unexpected results is commensurate in scope with the claimed invention (*see, e.g., In re Kulling*, 14 U.S.P.Q.2d 1056, Fed Cir. 1990), as Examiner Crouch agreed at the interview.

The Examiner suggests in the Office Action three motivations to combine Lonberg and Tomizuka: first, to produce mice expressing different antibody isotypes; second, to produce mice expressing greater diversity; and third, to produce mice with higher levels of antibody production. However, none of these factors provide the necessary motivation to combine the teachings of the references, much less to select the claimed combination.

With respect to antibody isotype, as discussed at the interview and in the specification, that selection is made by cloning the V region generated in the transgenic mouse into the desired constant region to achieve the desired isotype. Even with a full complement of heavy chain constant region genes present in the mouse, indeed because of that, isotype selection is uncontrollable. Combining a desired constant region isotype with a V-determined antigen specificity can only be controlled after cloning the antibody genes. This is not a motivation to combine Lonberg and Tomizuka because the combined references simply cannot address the problem.

With respect to repertoire diversity, again the combination works against the result. The greatest diversity arises from the double transchromosomal mice, which have a complete heavy

[1] Indeed, as discussed at the interview, one of the leading figures in transgenic mice for making human antibodies refused to accept the results even after the inventors achieved them.

chain and light chain loci. Any combination with the Lonberg miniloci would limit, not expand diversity.

Finally, the expectation of higher levels of antibody production is no motiviation either. First, the levels of antibody production in the transgenic animals is, as discussed at the interview, irrelevant. The transgenic animals are a tool to produce an antibody product lead. Production of the antibody for testing and commercial purposes involves, typically, recombinant expression systems. That is not a motivation to combine the references.

Although the Office Action does not state it this way, the higher level of B cell and antigen-specific B cell generation, as reflected in the hybridoma and subclone data in Tables 1 (page 65) and 2 (page 66), could not have been expected, as set forth above. Unexpected results such as these cannot form a motivation to combine references.

Lonberg *et al.* and Tomizuka *et al.*, taken alone or in combination, fail to teach or suggest the claimed invention. Lonberg *et al.* teach the use of *transgenes* to generate transgenic nonhuman animals capable of producing a heterologous antibody, such as human antibody. As set forth above, Tomizuka *et al.* teach a mouse wherein some mouse cells retain a *foreign chromosome* or fragment thereof that is maintained independent of the mouse chromosomes (*e.g.*, *transchromosome*), wherein the foreign chromosome or fragment thereof comprises a human antibody gene. Both Lonberg *et al.* and Tomizuka *et al.* successfully produced animals carrying heterologous genetic material encoding heterologous antibodies and these animals successfully make heterologous antibodies. Tomizuka *et al.* is silent with regard to the use of a transgene and to the use of a transchromosome in combination with a transgene. Similarly, Lonberg *et al.* is concededly silent with regard to the use of a transchromosome, and to the use of a transgene in combination with a transchromosome. The mere fact that the teachings of the prior art *can* be combined or modified, or that a person having ordinary skill in the art is capable of combining or modifying the teachings of the prior art, does not make the resultant combination *prima facie* obvious, as the prior art must also suggest the desirability of the combination (*see, e.g.*, *In re Mills*, 16 U.S.P.Q.2d 1430, Fed. Cir. 1990; *In re Fritch*, 23 U.S. P.Q.2d 1780, Fed. Cir. 1992). In this case, even though the combined references might contain each element of the claimed invention,

there is no motivation to combine them and *prima facie* obviousness does not apply. *In re Rouffet*, 149 F.3d 1350, 1357 (Fed. Cir. 1998).

In sum, Applicants respectfully submit that the Office Action has failed to set forth a *prima facie* case of obviousness. In particular, no evidence has been provided that, at the time of filing the instant application, a person having ordinary skill in the art would have been motivated to arrive at the claimed invention. Accordingly, Applicants respectfully request that this rejection be withdrawn.

REJECTION UNDER OBVIOUSNESS-TYPE DOUBLE PATENTING

In the Office Action, claims 1 and 6-8 were rejected under the judicially created doctrine of obviousness-type double patenting as unpatentable over claims 22, 25, 27-29, 32, 35, 36, 39-41, and 44-52 of U.S. Patent No. 6,632,976 (Tomizuka *et al.*).

Applicants respectfully traverse the ground of rejection. For the same reasons set forth above under "Rejection Under 35 U.S.C. §103(a)", Applicants submit that Tomizuka *et al.* do not teach or suggest the instant invention and, therefore, a *prima facie* case of obviousness has not been set forth. Instead, the Examiner reasoned that "... the claims of the cited patent [Tomizuka] encompass [the] instant claims." However, obviousness-type double patenting requires an analysis that parallels of a 103 obviousness rejection. See MPEP 804.B.1. citing *In re Braat*, 937 F.2d 589 (Fed. Cir. 1991) and *In re Longi,* 759 F.2d 881 (Fed. Cir. 1985). The Examiners agreed at the interview that a double patenting rejection over the '976 patent does not apply here (See the Examiner Interview Summary Record). Accordingly, Applicants respectfully submit that this rejection should be withdrawn.

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to pass this application to issue.

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Respectfully submitted,

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A transgenic mouse that expresses a diversity of human sequence heavy and light chain immunoglobulins

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ABSTRACT

We have generated transgenic mice that express a diverse repertoire of human sequence immunoglobulins. The expression of this repertoire is directed by light and heavy chain minilocus transgenes comprised of human protein coding sequences in an unrearranged, germ-line configuration. In this paper we describe the construction of these miniloci and the composition of the CDR3 repertoire generated by the transgenic mice. The largest transgene discussed is a heavy chain minilocus that includes human μ and $\gamma 1$ coding sequences together with their respective switch regions. It consists of a single 61 kb DNA fragment propagated in a bacterial plasmid vector. Both human heavy chain classes are expressed in animals that carry the transgene. In light chain transgenic animals the unrearranged minilocus sequences recombine to form VJ joints that use all five human J_κ segments, resulting in a diversity of human-like CDR3 regions. Similarly, in heavy chain transgenics the inserted sequences undergo VDJ joining complete with N region addition to generate a human-like V_H CDR3 repertoire. All six human J_H segments and at least eight of the ten transgene encoded human D segments are expressed. The transgenic animals described in this paper represent a potential source of human sequence antibodies for *in vivo* therapeutic applications.

INTRODUCTION

A number of investigators have previously reported immunoglobulin and T-cell receptor gene sequence rearrangements in transgenic animals. Bucchini *et al.* (1) reported the rearrangement of a germline chicken λ light chain locus inserted into transgenic mice. Similarly, Goodhart *et al.* (2) observed the rearrangement of a rabbit kappa light chain construct in mice. Two other groups (3,4) have made transgenic animals with chimeric rearrangement test constructs and shown that the transgenes rearrange during lymphoid cell development. Bruggemann *et al.* (5) generated mice containing a hybrid human/mouse heavy chain minilocus construct. This construct included one human and one mouse V segment, three mouse D segments (two of which had been altered by site directed mutagenesis to appear human), one human D segment, all six human J segments, and a chimeric human/mouse μ gene. The authors observed rearrangement of the transgene sequences in spleen and thymus as well as serum expression of human μ epitopes. No analysis of the structure of the rearrangements was reported. Bruggemann *et al.* subsequently reported the generation of transgenic mice by the co-injection of two cosmid clones (6). Together, these clones encompassed 100 kb of the human heavy chain locus and included most of the D region as well as the entire human J and μ regions. The constructs included 2 functional human V segments. The authors observed rearrangement of the transgene in lymphoid tissue; however, sequence analysis of the resulting VDJ joints showed only short CDR3 sequences with no recognizable human D segments.

In this report we describe transgenic animals that carry both light and heavy chain minilocus constructs comprised of human coding sequences. The heavy chain construct encodes two different isotypes, μ and $\gamma 1$. The transgenic mice that we have generated express a diversity of human sequence immunoglobulins that incorporate all of the human $J\kappa$ and J_H segments, at least eight different human D segments, and two different human heavy chain constant region segments. The human light and heavy chain CDR3 repertoires of these animals are comparable with authentic human CDR3 repertoires.

MATERIALS AND METHODS

Plasmid vectors

For the purpose of building very large transgene constructs in bacterial plasmids, we have developed a series of new cloning vectors. These vectors contain different polylinker sequences cloned into the NotI site of pGP1a, the first vector in the series. We generated pGP1a by ligating two synthetic oligonucleotides, caa gag ccc gcc taa tga gcg ggc ttt ttt ttg cat act gcg gcc gct and aat tag cgg ccg cag tat gca aaa aaa agc ccg ctc att agg cgg gct, into EcoRI/StyI digested pBR322. The resulting plasmid, pGP1a, is designed for cloning very large DNA constructs that can be excised by the rare cutting restriction enzyme NotI. It contains a NotI restriction site downstream (relative to the ampicillin resistance gene, AmpR) of a strong transcription termination

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signal derived from the trpA gene (7). The vectors pGP1b, pGP1c, pGP1d, and pGP1f were derived from pGP1a and contain different polylinker cloning sites. The polylinker sequences are: pGP1a, GCG GCC GC; pGP1b, GCg gcc gcc tcg aga tca cta tcg att aat taa gga tcc agc agt aag ctt gcG GCC GC; pGP1c, GCg gcc gca tcc cgg gtc tcg agg tcg aca agc ttt cga gga tcc gcG GCC GC; pGP1d, GCg gcc gct gtc gac aag ctt atc gat gga tcc tcg agt gcG GCC GC; pGP1f, GCg gcc gct gtc gac aag ctt cga att cag atc gat gtg gta cct gga tcc tcg agt gcG GCC GC. The heavy chain minilocus constructs were built in a plasmid vector derived from pGP1b that also contains the rat immunoglobulin 3′ heavy chain enhancer (8). This enhancer was amplified from rat liver DNA using the following two synthetic oligonucleotides as primers: ctc cag gat cca gat atc agt acc tga aac agg gct tgc and ctc cag gat cca gat atc agt acc tga aac agg gct tgc. The amplified product was digested with BamHI and SphI and cloned into BamHI/SphI digested pNNO3 (R. Tizard, Biogen, Cambridge, MA), a pUC derived plasmid that contains a polylinker with the following restriction sites, listed in order: NotI, BamHI, NcoI, ClaI, EcoRV, XbaI, SacI, XhoI, SphI, PstI, BglII, EcoRI, SmaI, KpnI, HindIII, and NotI. The resulting plasmid, pRE3, was digested with BamHI and HindIII, and the insert containing the rat Ig heavy chain 3′ enhancer cloned into BamHI/HindIII digested pGP1b to generate pGPe.

Heavy chain miniloci

Isolation of human μ sequences. We screened a λ phage library of human genomic DNA sequences with human μ specific oligonucleotide probes and isolated clones that spanned the J-μ-δ region. We combined three different fragments to generate the plasmid pJM2: a 6 kb HindIII/KpnI fragment containing all six J segments as well as D-segment DHQ52 and the heavy chain J-μ intronic enhancer, the adjacent downstream 10.5 kb HindIII/XhoI fragment, containing the μ switch region and all of the μ constant region exons, and a 4 kb XhoI fragment that contains sequences downstream and includes the so-called Σμ element involved in μ deletion in certain IgD expressing B cells (9, 10).

Isolation of human D region sequences. We used human D region specific oligonucleotides to isolate phage clones containing the D1 and D2 portions of the human D region. A 5.5 kb XhoI fragment, that includes the D elements D_{K1}, D_{N1}, D_{IR2}, D_{M2}, and D_{LR2} (11), was combined with the adjacent upstream 5.2 kb XhoI fragment that includes the D elements D_{LR1}, D_{XP1}, $D_{XP'1}$, and D_{A1}, to give the plasmid pDH1. pDH1 and pJM2 were combined to create the plasmid pCOR1. Plasmid pCOR1 was partially digested with XhoI and a 10.3 kb genomic HindIII fragment containing the functional human heavy chain variable region segments V_H251 and the variable segment pseudogene V_H105 (12) inserted upstream to produce the transgene construct pIGM1 (Figure 1). The plasmid pIGM1 contains a single functional human variable region segment, at least 10 human D segments, all 6 human J_H segments, the human J-μ enhancer, the human sm element, the human μ switch region, all of the human μ coding exons, and the human Sm element, together with the rat heavy chain 3′ enhancer.

Isolation of γ1 constant region sequences. We isolated human γ1 genomic clones from a phage library using specific oligonucleotide probes and confirmed by DNA sequence analysis that the clones belonged to the γ1 subclass. We combined three adjacent genomic fragments, a 5.3 kb HindIII fragment, a 7.6 kb HindIII/BamHI fragment, and a 4.5 kb BamHI fragment to generate the plasmid clone pγe2. pγe2 contains all of the γ1 constant region coding exons, and the upstream switch region and sterile transcript exons, together with 5 kb of downstream sequences, linked to the rat heavy chain 3′ enhancer. This clone contains a unique XhoI site at the 5′ end of the insert. The plasmid pIGM1 was digested with XhoI and the 43 kb insert isolated and cloned into XhoI digested pγe2 to generate the plasmid pHC1 (Figure 1).

Light chain minilocus

$V_κ$ gene. We screened a human genomic DNA phage library with the $V_κ$ light chain specific oligonucleotide probe 5′- agg ttc agt ggc agt ggg tct ggg aca gac ttc act ctc acc atc agc -3′ and isolated clones containing human $V_κ$ segments. To identify functional genes we determined the nucleotide sequence of several of the clones and looked for TATA box sequences, open reading frames encoding leader and variable peptides (including 2 cysteine residues), splice sequences, and recombination heptamer-12 bp spacer-nonamer sequences. The light chain construct that we describe in this paper contains a single $V_κ$-III segment ($V_κ$ 65.8) isolated from phage clone 65.8. The sequence of this gene is identical to that of a previously reported germline human $V_κ$ gene that appears to encode the light chain variable sequence of several reported IgM anti-IgG autoantibodies (13). This gene (HUMIGVA27) has been mapped to the Ab region of the human light chain locus (14).

pKC1. We screened a human genomic DNA phage library with κ light chain specific oligonucleotide probes and isolated clones spanning the J-$C_κ$ region. We cloned a 5.7 kb ClaI/XhoI fragment containing $J_κ1$ together with a 13 kb XhoI fragment containing $J_κ2-5$ and $C_κ$ into pGP1d to create the plasmid pKcor. This plasmid contains $J_κ1-5$, the κ intronic enhancer and $C_κ$ together with 4.5 kb of 5′ and 9 kb of 3′ flanking sequences. It also has a unique 5′ XhoI site for cloning $V_κ$ segments and a unique 3′ SalI site for inserting additional cis-acting regulatory sequences. The κ light chain minilocus transgene pKC1 (Figure 1) was generated by inserting a 7.5 kb XhoI/SalI fragment containing $V_κ$ 65.8 into the 5′ XhoI site of pKcor. The transgene insert was isolated by digestion with NotI prior to injection.

Generation of transgenic mice

We isolated the NotI inserts of plasmids pIGM1, pHC1, and pKC1 away from vector sequences by agarose gel electrophoresis. We then microinjected the purified inserts into the pronuclei of fertilized (C57BL/6×CBA)F2 mouse embryos and transferred the surviving embryos into pseudopregnant females as described by Hogan *et al.* (15). We analyzed the mice that developed from injected embryos for the presence of transgene sequences by Southern blot hybridization of tail DNA. We obtained 2 independent lines of mice containing the pIGM1 insert, 12 lines of mice containing the pHC1 insert, and 5 lines containing the pKC1 insert.

Serum analysis

We isolated serum from the blood of transgenic and non-transgenic animals and assayed for the presence of transgene encoded human Igκ, IgM and IgG_1 by ELISA as described by Harlow and Lane (16). Microtiter plate wells were coated with mouse monoclonal antibodies specific for human Igκ (clone 6E1, #0173, AMAC, Inc. Westbrook, ME), IgM (clone AF6,

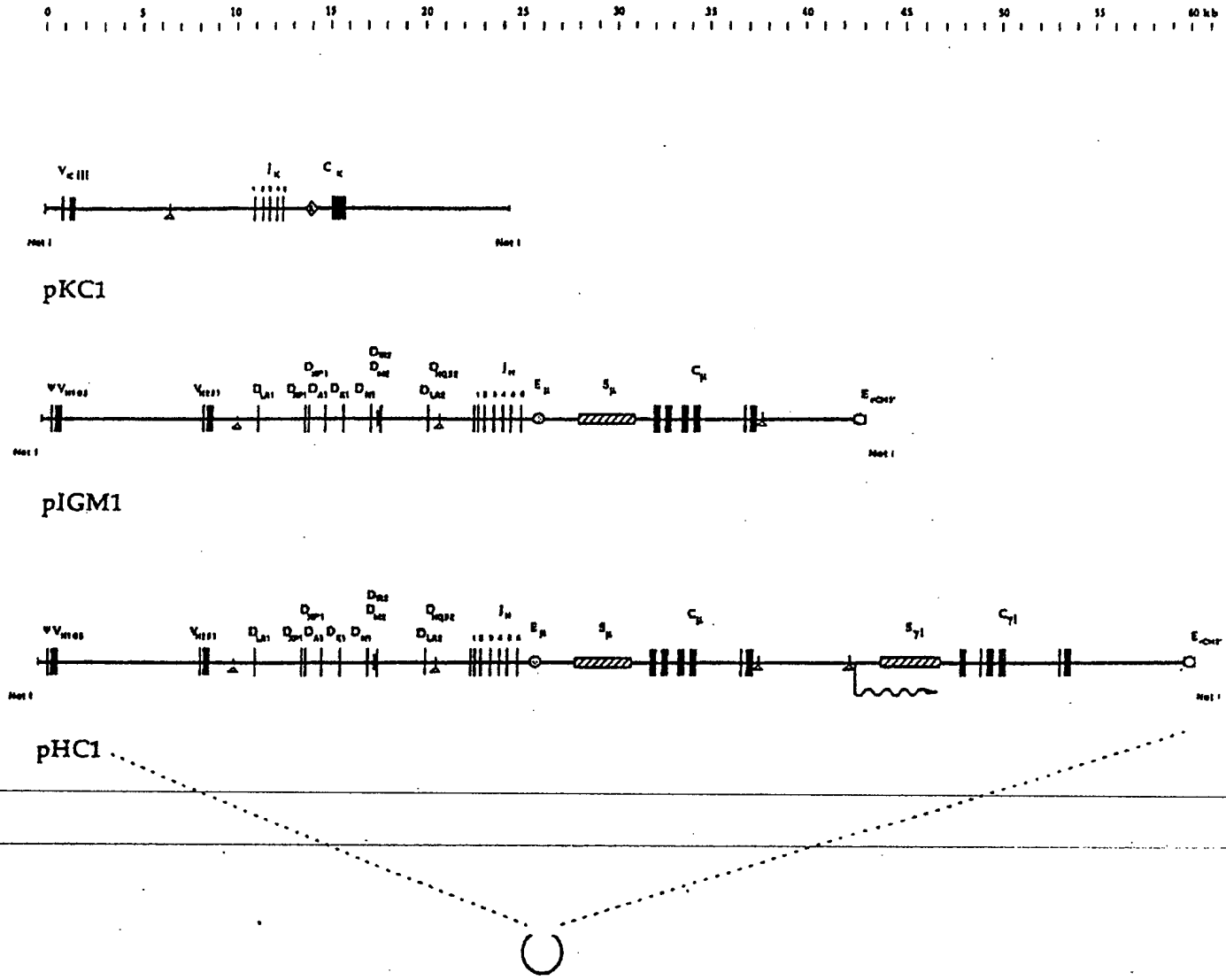

Figure 1. Human immunoglobulin minilocus transgene constructs. The three transgene inserts—KC1, IGM1, and HC1—are depicted as they appear prior to microinjection (after linearization with the restriction enzyme NotI and isolation from vector sequences). The open triangles indicate discontinuities between the structure of the transgene and the natural chromosomal structure of the intact human gene loci. The start site of the human γ1 pre-switch sterile transcript is indicated by the wavy arrow below HC1. V, variable segment; D, diversity segment; J, joining segment, C, constant region gene; S, switch region; E, enhancer.

#0285, AMAC, Inc. Westbrook, ME) and human IgG_1 (clone JL512, #0280, AMAC, Inc. Westbrook, ME). Serum samples were serially diluted into the wells and the presence of specific immunoglobulins detected with affinity isolated alkaline phosphatase conjugated goat anti-human Ig (polyvalent) that had been pre-adsorbed to minimize cross-reactivity with mouse immunoglobulins. We used monoclonal human IgG_1,κ (#0575, AMAC, Inc. Westbrook, ME) and human IgM (#009-000-012, Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) as standards.

cDNA clones

To assess the functionality of the pHC1 transgene in VDJ joining and class switching we examined the structure of immunoglobulin cDNA clones derived from transgenic mouse spleen mRNA. We isolated pA$^+$ RNA from the spleens of transgenic mice (17) and used this RNA to synthesize oligo-dT primed single stranded cDNA (18). The resulting cDNA was then used as template for PCR amplifications using the following synthetic oligonucleotides as primers: VH251 specific oligo-149, cta gct cga gtc caa gga gtc tgt gcc gag gtg cag ctg (g,a,t,c); human γ1 specific oligo-151, ggc gct cga gtt cca cga cac cgt cac cgg ttc; and human μ specific oligo-152, cct gct cga ggc agc caa cgg cca cgc tgc tcg. We isolated the resulting 0.5 kb PCR products from an agarose gel, digested with XhoI and cloned the fragments into the plasmid pNNO3. We determined the nucleotide sequences of the inserts by the dideoxy chain-termination method and compiled the data using the GeneWorks sequence analysis software (IntelliGenetics, Mountain View, CA).

Flow cytometry

We prepared single cell suspensions of splenocytes by crushing the spleens between frosted glass slides and lysing the red cells in NH_4Cl (19). Lymphocytes were stained with the following

Table 1. Transgenic founder animals generated with the KC1, IGM1, and HC1 miniloci

transgene	line #	~ copy #	serum expression
KC1	665	10–50	κ
	670	1–2	–
	673	1–2	κ
	674	10–50	κ
	676	5–20	κ
IGM1	6	10–50	μ
	15	5–20	μ
HC1	19	1–2	–
	21*	<1–	
	26	5–20	μ, γ1
	29**	>100	n.d.
	38	5–20	μ, γ1
	57	10–50	μ, γ1
	58	10–50	n.d.
	112	1–2	μ
	117	5–20	μ, γ1
	118	5–20	μ, γ1
	119	10–50	μ, γ1
	122	10–50	μ, γ1

Each transgenic line is designated by the I.D. # of the founder animal that developed from a microinjected embryo. The approximate number of copies of the inserted transgene is estimated by the intensity of the southern blot hybridization signal. Expression of human κ, μ, and γ1 epitopes was determined for most of the lines by ELISA of serum from either the founder animal or one of its descendants (n.d.: experiment not done; * mosaic; no positive offspring; ** hydrocephalic, died at 6 weeks).

reagents: biotin conjugated anti-human IgM (clone G20-27; Pharmingen, San Diego, CA), FITC conjugated anti-human IgM (clone G20-27; Pharmingen, San Diego, CA), FITC conjugated anti-mouse IgM (clone R6-60.2; Pharmingen, San Diego, CA), biotin conjugated anti-human-Igκ (clone G20-361; Pharmingen, San Diego, CA) and Cy-Chrome conjugated anti-mouse B220 (clone RA3-6B2; Pharmingen, San Diego, CA). Biotin conjugated reagents were then stained with phycoerythrin conjugated streptavidin (Becton Dickinson, San Jose, CA). Stained cells were analyzed using a FACscan flow cytometer and LYSIS II software (Becton Dickinson, San Jose, CA). Macrophages and residual red cells were excluded by forward and side scatter.

RESULTS

Human immunoglobulin minilocus constructs

To generate minilocus transgenes we have constructed large plasmid inserts assembled from multiple disparate chromosomal segments. That assembly required serial cloning steps, with the difficulty increasing at each step as the size and sequence complexity increased. To simplify this assembly process, we began by generating a new set of vectors specifically designed for building large transgenes. These vectors (pGP1a, pGP1b, pGP1c, pGP1d, pGP1f, and pGPe) are pBR322-based plasmids that are maintained at a lower copy number per cell than the pUC vectors (20). The vectors also include a strong transcription termination signal derived from the trpA gene (7). This termination signal should reduce the potential toxicity of coding sequences inserted into the NotI site by eliminating read-through transcription from the ampicillin resistance gene. In addition, these vectors contain polylinkers that are flanked by restriction sites for the rare-cutting enzyme NotI; thus allowing for the isolation of the insert away from vector sequences prior to embryo microinjection.

Figure 1 depicts one light chain minilocus construct and two heavy chain minilocus constructs that we have used to generate transgenic animals. The light chain transgene, pKC1, consists of a single Vκ-III family variable segment, all five human Jκ segments, and the human Cκ segment, together with 8 kb of downstream sequences. The entire 25 kb transgene insert can be isolated using the restriction enzyme NotI. The first heavy chain transgene, pIGM1, consists of a single functional V_H-V family variable segment, ten different human D segments, all six human heavy chain J segments, the μ switch region, and the entire human μ coding region. In addition the construct includes the rat heavy chain 3′ enhancer. The 43 kb transgene insert can be isolated using the restriction enzyme NotI. The final construct, pHC1, is identical to pIGM1 except for the insertion, after the μ gene, of an additional 18 kb of sequence that includes the human γ1 gene and switch sequences. This construct includes the transcription start site for the sterile transcript associated with isotype switching to γ1 (21). Like the two other transgene inserts this 61 kb transgene insert can also be isolated using the restriction enzyme NotI. We isolated and microinjected each of these three transgene inserts into mouse embryo pronuclei and generated a total of 19 transgene-positive founder animals (table 1).

Detection of human-sequence immunoglobulins in the serum

We collected serum samples from transgenic and control non-transgenic littermates and looked for the expression of human Igκ, μ, and γ1 epitopes by ELISA. All of the control non-transgenic mice tested negative for serum expression of human Igκ, μ, and γ1 epitopes by this assay. Mice from the two lines containing the pIGM1 NotI insert (lines #6 and 15) express human μ. We tested mice from ten lines that contain the pHC1 insert and found that one of the lines (line # 112) expresses low levels of human μ but no detectable human γ1, seven of the lines (lines #26, 38, 57, 117, 118, 119, and 122) express both human IgM and human IgG1, while mice from two of the lines (lines #19 and 21) do not express detectable levels of human immunoglobulins. Expression levels varied between lines and between individual mice, with mice derived from the multi-copy lines #26 and 57 expressing the highest levels. These mice express human IgM and IgG1 at levels ranging from 0.1 to 1 microgram/ml. Of the three HC1 lines that did not express both transgene encoded isotypes and the one KC1 line that did not express human κ, all were either low copy, mosaic or both. One of these non-expressing lines (#21) was a mosaic that did not pass the transgene on to its offspring. It is possible that transgene-containing cells did not populate the hematopoietic lineage in significant numbers. Two of the lines (#19 and #670) appear by southern blot hybridization intensity to contain only one or two copies of the transgene. The transgene inserts may not be full length in these lines, or the level of expression may be below that which is detectable by our assay. Similarly, line HC1–112, which expresses μ but not γ1, may be missing 3′ transgene sequences necessary for γ1 expression.

Cell surface expression of transgene encoded immunoglobulins

We isolated spleen and peripheral blood lymphocytes from eight different lines of transgenic mice; two lines containing the light chain transgene, KC1, and six lines containing the heavy chain

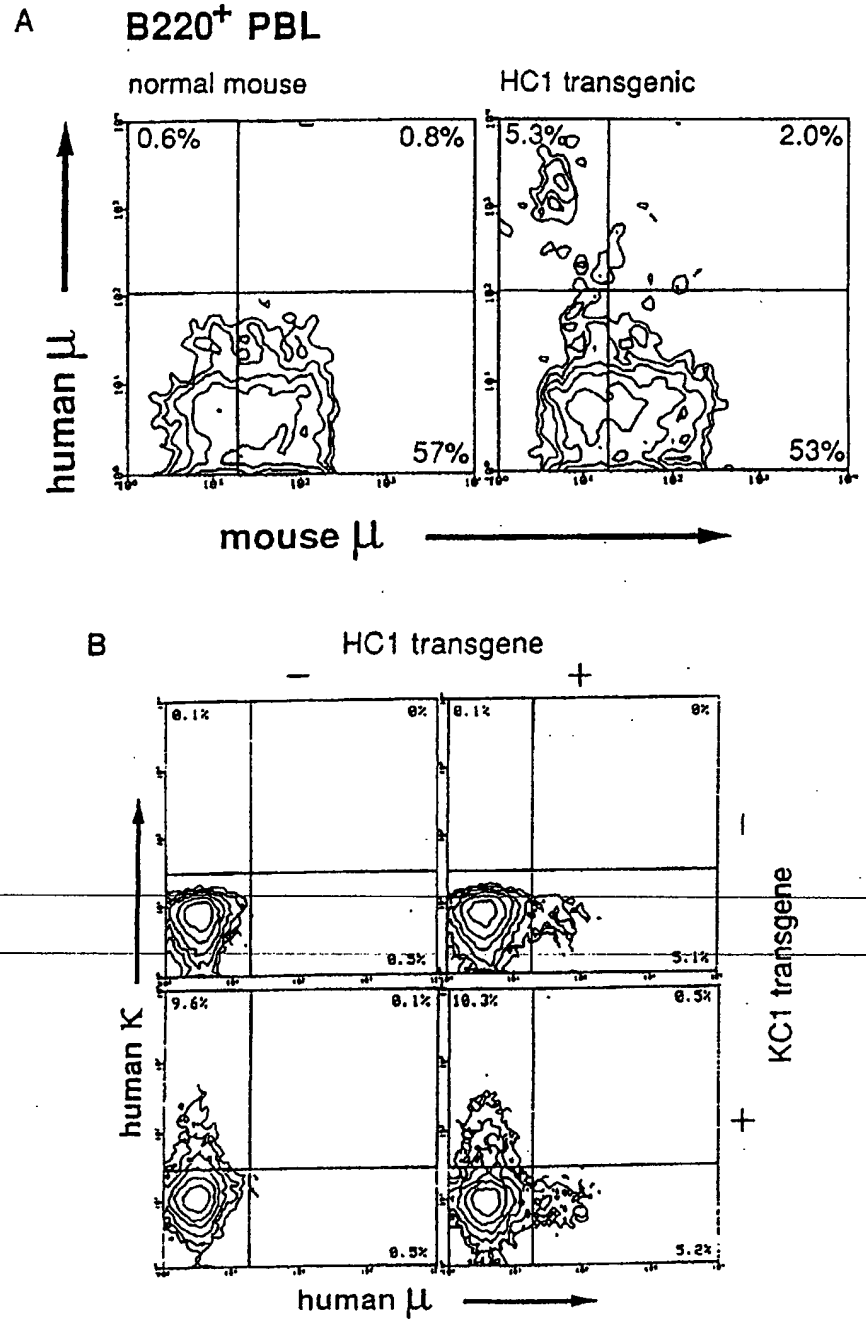

Figure 2. Detection of human sequence immunoglobulins on the surface of transgenic B cells by flow cytometric analysis. (A) Peripheral blood lymphocytes from a negative control and an HC1 line 26 transgenic animal gated for expression of the mouse B cell antigen B220 and assayed for mouse μ (FITC, x-axis) and human μ (PE, y-axis). (B) Spleen lymphocytes from four littermates: upper left, # 1072, HC1-57 negative; KC1-674, negative; upper right, # 1074, HC1-57 positive; KC1-674, negative; lower left, # 1069, HC1-57 negative, KC1-674, positive; lower right, # 1073, HC1-57 positive, KC1-674, positive. Cells were gated for expression of the mouse B cell antigen B220 and assayed for expression of human μ (FITC, x-axis) vs. human κ (PE, y-axis). Cell numbers are indicated by contour lines generated using LYSIS II software (Becton Dickinson, San Jose, CA). Number of cells in each quadrant is given as a percent of the B220 positive/lymphocyte scatter gate.

transgene, HC1. A fraction of the lymphocytes from each of these lines expressed human sequence immunoglobulins on their surfaces as assayed by fluorescent antibody staining and flow cytometry. The percentage of B cells expressing the transgene encoded products varied from 1–2% (for the single copy heavy chain line HC1-112) to 10–20% (for the multi-copy heavy chain line HC1-122). This is illustrated by the example shown in Figure 2A. We isolated peripheral blood lymphocytes from an HC1–26 transgenic animal and a negative littermate, and looked for expression of mouse and human μ heavy chain. In both animals the majority of the peripheral blood B cells express the mouse μ heavy chain; however, a fraction of the cells in the transgenic animal express the human μ chain, and a majority of these cells (5% of the total B220 positive cells) are mouse μ dull

Figure 4. Transgene encoded heavy chain CDR3 sequence diversity. The nucleotide and translated amino acid sequence of the junctional region of 49 independent cDNA clones is shown. Out of frame VDJ joints are not translated. Two of the D segment assignments (clones 215−15 and 883−95) are based on only 5 nucleotides of homology, and therefore represent possible assignments. All other assignments are based on greater than 5 nucleotides of homology. Nucleotides assigned to N regions are in lower case letters. Each clone is identified by two numbers separated by a dash; the first number indicates the ID # of the animal that provided the RNA, and the second number specifies the clone. Animal #215 was a heavy chain minilocus transgenic derived from line HC1-57. Animal #883 was a double (heavy and light chain minilocus) transgenic derived from lines HC1-26 and KC1-665 (light chain sequences from this animal are shown in Figure 4). Animal #640 was a heavy chain minilocus transgenic derived from the single copy line HC1-112. Clones 215-5 and 215-17, and clones 640-8 and 640-3.2 are identical.

Because we do not observe nucleotide changes elsewhere, we interpret these to be the result of random nucleotide additions introduced during VJ joining, and not later somatic mutations occurring during B cell maturation.

Heavy chain CDR3 sequences

Figure 4 shows the nucleotide sequences of human heavy chain CDR3 sequences derived from 49 individual cDNA clones from three different transgenic animals. We identified 47 unique cDNA sequences from these 49 clones. 36 of the 49 clones represented in-frame VDJ joints. This sampling shows that, as observed for the light chain minilocus, the expressed human heavy chains also represent a diverse repertoire and not a mono- or oligoclonal expansion of a limited set of rearrangements. Both μ and $\gamma 1$ sequences are represented. All six human J_H segments are incorporated, and eight of the ten transgene encoded human D segments are found in heavy chain transcripts. Also as observed for the light chain clones, there was no evidence of somatic mutation in the heavy chain sequences. Essentially all of the non-germline encoded nucleotides occurred at V-D, D-J, or V-J junctions and could be ascribed to N region addition. The frequency of non-germline encoded nucleotides outside of N regions is approximately 0.2% (data not shown) and may be the result of errors introduced by reverse transcription and PCR amplification. Because none of the mice had been immunized or exposed to pathogens (all animals were housed in micro isolator cages and were healthy) it is not surprising that we find no evidence of somatic hypermutation.

DISCUSSION

Xenotypic exclusion

We have shown by flow cytometric analysis that most of the human IgM-expressing B cells in our transgenic animals express at most low levels of endogenous mouse IgM. This suggests that correct rearrangement of the human transgene is capable of excluding the rearrangement of the mouse heavy chain locus. Confirmation will require structural analysis of the endogenous loci from a statistically significant number of hybridomas expressing the transgene. However, Nussenzweig *et al.* (23) reported exclusion of endogenous μ expression in transgenic mice containing a rearranged human μ gene. Rearrangement exclusion appears to depend on the expression of the transmembrane form of the heavy chain (24, 25) and presumably requires that it forms a functional complex with the products of the B29 and mb-1 genes (26, 27). Therefore, the xenotypic exclusion implied by our data and that of others suggests that the human heavy chain is capable of forming a functional complex together with the endogenous mouse non-IgH components of the receptor, and that this hybrid complex can induce B cell maturation beyond the developmental stage during which VDJ joining takes place.

Light chain CDR3 sequence analysis

The light chain minilocus encoded transcripts are diverse and incorporate all five human Jκ segments. Approximately one quarter of the Vκ-Jκ joints include non-germline encoded sequences. This addition of junctional random nucleotides is commonly associated with heavy chain N regions (28, 29). The large number of naturally occurring Vκ segments makes it difficult to determine whether or not N region addition is a normal component of κ light chain VJ joining (30 - 33); however, because the KC1 minilocus construct contains only a single variable segment, the transgenic result is unambiguous. Similar N region additions have been reported previously in light chain transgene rearrangements (34). It is possible that the abnormal chromosomal location of the transgene or the concatenated structure of the integrated locus could lead to premature rearrangement accompanied by N region addition. Alternatively, limited N region addition may be a normal component of light chain rearrangement that is difficult to recognize beneath the usual diversity of κ variable segments and somatic mutations. Whether or not the observed light chain N regions are an artifact of the transgenic system, they do not lead to abnormally long CDR3 sequences because the additions are compensated for by exonucleolytic reduction of the V and J segments. Six of the seven transcripts with N region additions result from in-frame VJ joints. Of these, five produce a ten amino acid CDR3 (the expected length given exact V-J joining with no exonucleolytic activity) and the sixth generates a nine residue CDR3. Furthermore, out of all of the 27 in frame transcripts we analyzed, 15% have 8 residue CDR3 sequences while 52% have 9 residue and 19% have 10 residue CDR3's. In comparison, analysis of the 34 naturally occurring Vκ-III nucleotide sequences reported by Kabat (35), shows that 12%, 71%, and 15% have 8, 9, and 10 residue CDR3's respectively. Therefore, N region addition does not appear to skew the size distribution of the light chain CDR3's away from that of an authentic human repertoire.

Heavy chain CDR3 sequences

Incorporation of J and D segments. The heavy chain minilocus-encoded transcripts are also diverse and incorporate all 6 human J_H segments, at least 8 of the 10 human D segments, and both heavy chain isotypes included in the transgene. We compared the human heavy chain CDR3 sequences that we isolated from transgenic mice to naturally occurring human CDR3 sequences from published reports (36, 37). The transgenic mice preferentially use J_H4 (47%) followed by J_H6 (22%). Yamada *et al.* (37) found a similar pattern; 53% of the authentic human joints incorporate J_H4 and 22% incorporate J_H6. It is more difficult to compare D segment usage between the transgenic mice and human PBL because the transgene minilocus does not include all of the human D region. 48% of the 75 in-frame clones analyzed by Yamada *et al.* could be assigned to D segments included in the HC1 transgene, and a further 11% could not be assigned to any known human D segment. These CDR3's either consist almost entirely of N region additions flanking very short D segment remnants or incorporate previously unrecognized D genes. Given these constraints two observations can be made. First, the DXP family is the most heavily used in both the transgenic animals and in human PBL, accounting for 31% and 29% respectively of the in-frame sequences. The second observation is that while only one of the in-frame human PBL sequences used DHQ52, 33% of the in-frame transgenic sequences (25% of all transgenic sequences) used DHQ52.

N region addition. The average length of the CDR3 sequences encoded by the 36 in-frame transcripts from the transgenic animals is 10.6 amino acids. This is similar to the average CDR3 length of 10.3 residues found for adult PBL sequences by Sanz (36). However, the transgenic sequences are considerably shorter than the 14.5 residue average found by Yamada *et al.* (37) for adult PBL sequences. The length difference between the average naturally occurring heavy chain CDR3 and the sequences found in the transgenic animals is predominantly due to differences in

N region addition. The average number of N region nucleotides per CDR3 sequence (excluding from analysis those sequences for which no D segment could be assigned and thus the N-D border could not be established) is 5.7 for the transgenic sequences and 14.3 for the adult human sequences reported by Yamada *et al.* This average increase in N nucleotides adds approximately 3 amino acids to the authentic human sequences. It appears that 93% of the V-D and D-J junctions in the 88 D containing adult PBL genomic DNA clones reported by Yamada *et al.* include N regions, and that the average length of these individual N regions is 7.7 bp. In contrast, 77% of the heavy chain junctions formed in the transgenic mice include N regions with an average length of 3.8 bp. This is close to the average length of 3 bp/N region for the 63 adult mouse cDNA clones published by Feeney (38). Although the heavy chain CDR3 sequences appear superficially like a human fetal liver repertoire because of the overuse of DHQ52 and the shorter average size of the N regions (39), the transgenic sequences do not resemble mouse fetal gene rearrangements which are more dramatically reduced in N region addition than human fetal rearrangements. Feeney found that the frequency of N region containing genomic clones fell from 83% in the adult to below 2% in the fetal liver. Therefore, we interpret the fetal character of the CDR3's to be a consequence of mouse B cell N nucleotide addition (which is less extensive than human) coupled with an increase in DHQ52 incorporation that may be peculiar to the transgene.

Implications for the generation of human sequence monoclonal antibodies

If B cells expressing human minilocus-encoded receptors are able to respond to antigen stimulation and undergo affinity maturation it will be possible to use the transgenic animals that we have generated as a source of human sequence monoclonal antibodies. This requires the functional replacement within the B cell receptor complex of the mouse heavy chain by the human heavy chain. It is therefore encouraging that we find B cells in the periphery of transgenic animals that express only the transgene-encoded human heavy chain, indicating that the human/mouse hybrid receptor is able to carry a mouse B cell through development. It is also important that rearrangement of the germline V, D, and J segments generates antibodies that resemble authentic human antibodies. We find that the light and heavy chain CDR3 sequences generated by rearrangement of the introduced miniloci fall within the range of authentic human CDR3 sequences.

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Human immunoglobulin transgenes undergo rearrangement, somatic mutation and class switching in mice that lack endogenous IgM

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Abstract

We have generated transgenic mice that contain human-sequence Ig miniloci and, because they are also homozygous for a targeted disruption of their endogenous heavy chain genes, must rely on the transgene sequences for B cell receptor expression. Although the human transgenes contain only a fraction of the intact human heavy chain locus, these defined sequences are able to at least partially restore the humoral immune system in the mouse. B cells expressing human heavy chains develop in the bone marrow, populate peripheral lymphoid tissue and respond specifically to antigen. Furthermore, the heavy chain transgenes contain both human μ and γ1 coding exons as well as the respective μ and γ1 switch regions. The sequences included within the transgene are sufficient to direct class switch recombination. Transgene sequences are also sufficient to direct somatic mutation of the class-switched heavy chain genes. These observations define the upper limit of the *cis*-acting sequences necessary to direct heavy chain class switching and somatic mutation.

Introduction

B cell development is dependent upon the expression of a functional cell surface receptor complex comprising Ig heavy and light chain proteins (1,2). These proteins are encoded by large, 2–3 megabase, genetic loci that undergo rearrangement during B cell development to generate a heterogeneous population of cells with different receptor specificities (3–6). This primary diversity is further expanded during a response to exogenous antigen by the process of somatic mutation (7–9). Exposure to antigen also leads to heavy chain class switching (10,11), thus producing antibody molecules with a variety of different effector functions. We and others have previously reported that relatively small transgene constructs, containing substantially less sequence information than the intact Ig loci, are able to rearrange during B cell development to generate a diversity of heavy and light chains (12–19). Others have also shown that mouse κ light chain transgenes can be generated that contain sufficient *cis*-acting sequences to direct somatic diversification of the encoded light chain (20–22). However, the sequences that direct somatic diversification of heavy chain (IgH) genes have not been previously located. Although it has been demonstrated that IgH transgenes undergo class switching to endogenous constant region genes (23) and that transgene sequences can be somatically mutated upon recombination into the endogenous IgH locus (24,25), these observations only exclude distant upstream sequences from involvement. In this report we show that sequences closely linked to coding exons autonomously direct the somatic diversification and class switching of IgH genes without fusion to endogenous constant region segments. Furthermore, we demonstrate that the transgene-encoded receptors function in B cell development and, despite an extremely limited heavy chain primary repertoire, the transgenic B cells participate in antigen specific responses.

It has been previously demonstrated that a rearranged mouse μ transgene can rescue B cell development in chimeric mice lacking a functional endogenous heavy chain locus (26). To demonstrate that a human sequence germline configuration minilocus can functionally replace the authentic locus, we bred a mouse strain lacking endogenous IgH with strains containing human germline-configuration IgH transgenes. The two transgene miniloci, HC1 and HC2, include one and four functional variable (V) segments respectively, 10 and 16 diversity (D) segments respectively, all six

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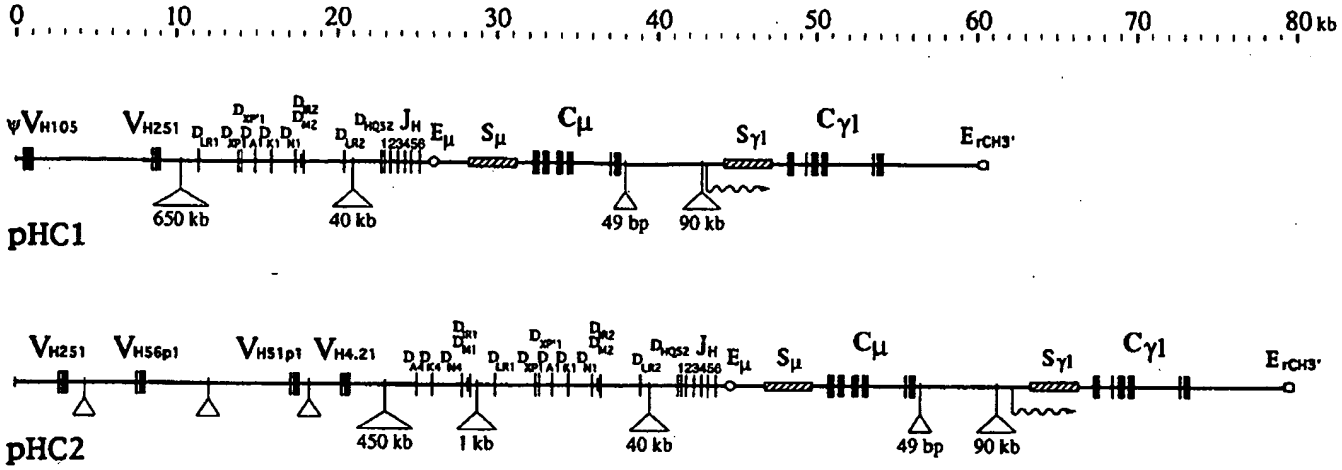

Fig. 1. Human Ig heavy chain minilocus transgenes. The pHC1 and pHC2 transgene inserts are depicted prior to microinjection (after linearization with the restriction enzyme *Not*I and isolation from vector sequences). The open triangles indicate discontinuities between the structure of the transgene and the natural chromosomal structure of the intact human heavy chain locus. The chromosomal distance spanning each discontinuity is shown below the triangles; distances are not given for the discontinuities separating the individual V segments in pHC2 because the segments are out of order relative to the natural human heavy chain locus. The start site of the human γ1 pre-switch sterile transcript is indicated by the wavy arrow below HC1. V, variable segment; D, diversity segment; J, joining segment; C, constant region gene; S, switch region; E, enhancer.

joining (J_H) segments and both the μ and γ1 constant region segments (16 and Fig. 1). All of these coding segments are human. The miniloci include human *cis*-acting regulatory sequences—such as the $J_H-\mu$ intronic enhancer and the μ and γ1 switch sequences—that are closely linked to the coding segments. They also include an additional enhancer element derived from the 3′ end of the rat IgH locus (27). We crossed HC1 and HC2 transgenic mice with stem-cell derived mutant mice that lack J_H segments (J_HD mice) and cannot therefore undergo functional heavy chain rearrangements (28). The resulting transgenic-J_HD mice contain B cells that are dependent on the introduced heavy chain sequences.

Methods

Transgene constructs

We constructed two different plasmids, pHC1 and pHC2, containing heavy chain minilocus transgenes. pHC1, which has been described previously (16), comprises a single functional variable gene segment, 10 functional D segments, all six J segments, the μ and γ1 constant region segments and the μ and γ1 switch regions. pHC1 also contains the human heavy chain $J-\mu$ intronic enhancer and the rat heavy chain 3′ enhancer. The entire 61 kb transgene can be isolated from plasmid vector sequences by digestion with *Not*I. The larger plasmid, pHC2, contains additional sequences not found in pHC1. pHC2 includes three additional variable gene segments, 5 additional D segments and 700 bp of additional sequence upstream of the γ1 switch region. The non-functional V_H pseudo-gene, V_H105, found in pHC1 is not included in pHC2.

To construct pHC2, we first combined four different germline configuration variable gene segments on a single plasmid. We isolated a germline configuration V_H1 family gene segment by screening a human placental genomic DNA phage library (Stratagene, La Jolla, CA) with the following synthetic oligonucleotide: 5′-GTT AAA GAG GAT TTT ATT CAC CCC TGT GTC CTC TCC ACA GGT GTC-3′. We determined the nucleotide sequence of an 800 bp segment of one of the positive phage clones. This sequence contains a functional V_H gene that is identical to the previously reported gene hv1263 (29). It is most likely an allele of the germline parent of the fetal cDNA clone 51p1 (30). We refer to the gene as V_H51p1. We subcloned a 6.3 kb *Xba*I fragment of the phage clone into the pUC derived plasmid pNNO3 (R. Tizard, Biogen, Cambridge, MA). The resulting plasmid, pVH49.8, contains vector polylinker derived *Cla*I and *Xho*I sites, 3′ and 5′ of the insert respectively.

The plasmid SHq32xx-4.6/pUC12 (R. Baer, University of Texas Health Center and T. H. Rabbitts, MRC, Cambridge) consists of a 4.6 kb *Xba*I fragment containing the germline V_H4 family gene V_H5 (31) cloned into the vector pUC12 such that the polylinker derived *Sma*I and *Hind*III sites are located 5′ and 3′ of the insert respectively. It appears to be an allele of the V_H4.21 gene described by Sanz *et al.* (32); homologous genes have also been designated DP-63 (33) and V4-34 (4). We refer to the gene as V_H4.21. We isolated the insert by digestion with *Sma*I and *Hind*III, thus attaching a polylinker *Sal*I site to the 3′ end, and cloned it by blunt-end ligation into the *Cla*I site of pV$_H$49.8 to create the plasmid pV2. This plasmid contains the human heavy chain gene V_H51p1 linked upstream of V_H4.21 in the same orientation, with unique *Xho*I and *Sal*I sites 5′ and 3′.

The plasmid p6.2 (J. D. Capra, University of Texas Southwestern) consists of a 7.7 kb *Eco*RI fragment containing the functional V_H3 family gene GL-SJ2 (34). The gene is most likely an allele of the germline parent of the fetal cDNA clone 56p1 (30). Homologous genes have been designated DP-46 (33), 3d216 (35) and V3-30 (4). We refer to the gene as V_H56p1. We subcloned the *Eco*RI fragment into the plasmid pGP1f (16), such that the *Xho*I and *Sal*I sites are at the 5′ and 3′ ends of the insert respectively, to generate p6.2f. We then cloned the *Xho*I–*Sal*I insert of pV2 into the 3′ *Sal*I site of p6.2f to generate pV3. This clone contains three functional V_H genes (V_H56p1, V_H51p1 and V_H4.21), in the same orientation, with *Xho*I and *Sal*I sites at the 5′ and 3′ ends of the insert respectively.

We subcloned a 4.2 kb *Sph*I – *Hin*dIII fragment containing the V_H5 family gene V_H251 (36,16) into the vector pSELECT-1 (Promega, Madison, WI) to attach a polylinker *Sal*I site to the 5′ end of the insert. We then subcloned the *Sal*I – *Hin*dIII insert into pSP72 to attach a polylinker *Xho*I site to the 3′ end. We inserted the *Sal*I – *Xho*I fragment of this clone into the 5′ *Xho*I site of pV3 to generate pV4. This plasmid contains four functional variable gene segments (V_H251, VH56p1, V_H51p1 and $V_H4.21$) in the same orientation on a single *Not*I – *Sal*I fragment.

To isolate additional human D segments, we screened a human genomic DNA phage library with the following synthetic oligonucleotide: 5′-CCT CGA TGG CAG GCG GAG AAG ATT CAG AAA GGT CTG AGA TCC-3′. We determined by DNA sequence analysis that one of the clones, λD21, contains the DA4, DK4 and DN4 diversity segments (37). We subcloned a 5.2 kb *Hin*dIII fragment of λD21, containing DA4, DK4, DN4, DIR1 and part of DM1, into pSP72 (Promega) such that the polylinker *Xho*I and *Sal*I sites flank the 5′ and 3′ ends of the insert respectively. We then eliminated the 5′ *Hin*dIII site by partial digestion, Klenow fill-in and re-ligation, to generate pD21csk. To complete the DM1 segment, we subcloned an adjacent 0.5 kb *Hin*dIII – *Xho*I fragment from λD21 into *Hin*dIII – *Sph*I digested pD21csk, destroying the *Xho*I and *Sph*I ends by fill-in ligation. The resulting clone, pDH3, contains the DA4, DK4, DN4, DIR1 and DM1 segments on a 5.7 kb fragment, flanked at the 5′ and 3′ ends by *Xho*I and *Sal*I. We cloned this fragment into the unique 3′ *Sal*I site of pV4 to generate pV4D.

We isolated a 0.7 kb *Xba*I – *Hin*dIII fragment from the phage clone $\lambda S_\gamma 1.13$, which we previously used to subclone the human γ1 switch region (16). This fragment contains sequence immediately upstream from the switch-containing 5.3 kb *Hin*dIII fragment incorporated in pHC1. We inserted the 0.7 kb *Xba*I – *Hin*dIII fragment into a plasmid that included pSP72 derived polylinker sequences encoding the sites *Hin*dIII, *Sph*I, *Pst*I and *Sal*I. These sites were attached to the 3′ end of the fragment and the 5′ *Xba*I end converted to a *Sal*I site by linker ligation. We then isolated the sequence as a 0.7 kb *Sal*I fragment and cloned it into pSP72 to attach an *Xho*I site to the 5′ end. We then isolated the fragment by *Sal*I-partial and *Xho*I-complete digestion and cloned it in the correct orientation into the unique 5′ *Xho*I site of $p_\gamma e2$ (16) to generate $p_\gamma e3$. The plasmid pge3 contains an 18 kb insert comprising the human γ1 switch region and constant region coding exons, together with 0.8 kb upstream of the first exon of the γ1 sterile transcript (38) and >5 kb downstream of the last coding exon (39) linked to the pGPe derived rat heavy chain 3′ enhancer (16,27). The plasmid includes a unique *Xho*I site followed by a unique *Sal*I site at the 5′ end of the insert; *Not*I sites flank the entire insert.

We cloned the 32 kb *Xho*I insert of the plasmid pCOR1—which includes the DLR1, DXP1, DXP′1, DA1, DK1, DN1, DIR2, DM2, DLR2 and DHQ52 segments, all six J segments, the μ enhancer and switch regions, together with the μ coding exons (16)—into the *Sal*I site of pge3 to generate pCOR2. We combined the *Xho*I – *Not*I insert of pCOR2 with the *Not*I – *Sal*I insert of pV4D into *Not*I digested pGP1b to generate the transgene minilocus construct pHC2. The entire 80 kb insert of pHC2 is contained on a single *Not*I fragment.

Transgenic mice

We generated pHC2 transgenic mice as previously described for pHC1 transgenics (16). We isolated the *Not*I insert of pHC2 from vector sequences by agarose gel electrophoresis. We then microinjected the purified 80 kb fragment into the pronuclei of half-day $(C57BL/6 \times CBA)F_2$ embryos and transferred the surviving embryos into pseudopregnant females as described by Hogan *et al.* (40). We analyzed the 109 mice that developed from injected embryos for the presence of transgene sequences by Southern blot hybridization of tail DNA and obtained 18 independent founder animals containing the HC2 minilocus transgene. Mice containing targeted deletions of the heavy chain J region are described elsewhere (28).

Southern blot hybridizations

We isolated genomic DNA by proteinase K digestion of tissue or cells, followed by phenol – chloroform extraction and isopropanol precipitation. Restriction enzyme digested DNA was separated on agarose by electrophoresis, acid/alkali nicked and denatured and blotted onto Nylon membranes in gel buffer. Blots were hybridized with ^{32}P-labeled probe at 65°C in 7% SDS, 65 mM Na_2HPO_4, 7 mM Na_4EDTA and 0.1% Triton X-100 titrated to pH 7.2 with H_3PO_4; and washed sequentially in undiluted, 5-fold and 10-fold diluted hybridization buffer (41).

cDNA clones

We prepared single-stranded cDNA from HC1 and HC2 transgenic spleen and lymph node (including axillary, inguinal and mesenteric) tissue as previously described (16) and amplified the transgene encoded cDNA sequences by polymerase chain reaction (PCR). The 5′ and 3′ primers for HC1 transgenic cDNA are described elsewhere (16). The mouse γ specific oligonucleotide is: 5′-GGC GCT CGA GCT GGA CAG GG(A,C) TCC A(G,T)A GTT CCA-3′. The 3′ μ and γ1 specific primers are the same for HC2 as HC1; however, the HC2 5′ primers consist of an equimolar mixture of the following four oligonucleotides: V_H251, 5′-CTC CAA GAA TTC TGT GCC GAG GTG CAG CTG GTG CA(G,A,T,C)-3′; V_H56p1, 5′-GCA AGA GAA TTC CAG TGT CAG GTG CAG CTG GTG GA(G,A,T,C)-3′; V_H51p1, 5′-GCT ACA GAA TTC CAG TCC CAG GTC CAG CTG GTG CA(G,A,T,C)-3′; $V_H4.21$, 5′-GCT CCC GAA TTC CTG TCC CAG GTG CAG CTA CAG CA(G,A,T,C)-3′. The amplified cDNA is digested with *Eco*RI and *Xho*I, cloned into plasmid vectors and sequenced by the dideoxy chain termination method. We compiled the DNA sequences using the GeneWorks sequence analysis software (IntelliGenetics, Mountain View, CA).

Immunizations and hybridomas

We immunized mice by i.p. injections of 50 – 100 mg of antigen. Antigens included human carcinoembryonic antigen (CEA; Crystal Chem, Chicago, IL), hen egg white lysozyme (Pierce, Rockford, IL) and keyhole limpet hemocyanin (KLH; Pierce, Rockford, IL). For primary injections we mixed the antigen with complete Freund's adjuvent, for subsequent injections we used incomplete Freund's adjuvent (Gibco BRL, Gaithersburg, MD). We fused spleen cells with the non-secreting mouse myeloma P3X63-Ag8.653 (ATCC, CRL1580). We assayed serum samples and hybridoma supernatants for the presence of specific and non-specific antibody comprising human heavy chain sequences by ELISA. For detection of non-specific antibodies we coated microtiter wells with human heavy chain isotype specific antibody (mouse mAb anti-human IgG1, clone HP6069, Calbiochem, La Jolla, CA; mouse mAb anti-human IgM, clone CH6, The Binding Site, Birmingham, UK) and developed with peroxidase conjugated antisera [horseradish peroxidase-conjugated affinity purified fab fragment from polyclonal goat a human IgG(fc), cat. no. 109 – 036 – 098; affinity purified

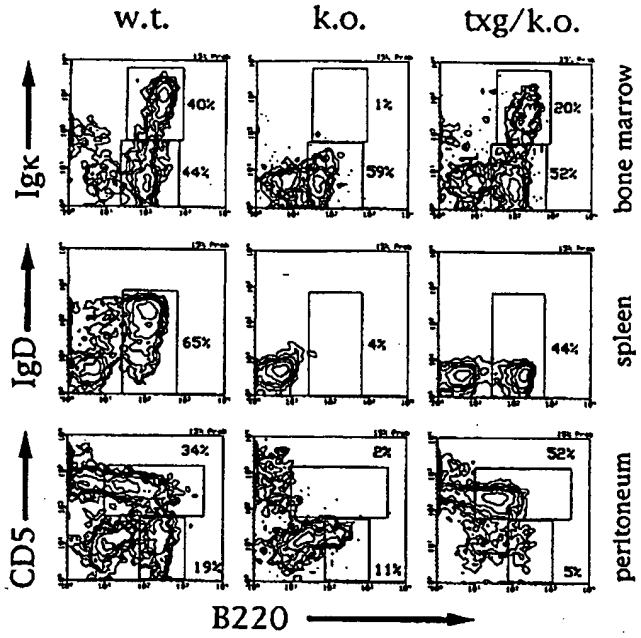

Fig. 2. Rescue of B cell compartments by human minilocus transgene. Flow cytometric analysis of fluorescent stained cells isolated from lymphoid tissues. Left column: control animals (wild-type heavy chain locus/non-transgenic). Center column: J_HD animals (homozygous for disrupted heavy chain locus). Right column: transgenic-J_HD animals (homozygous for disrupted heavy chain locus and hemizygous for HC1 line 57 transgene integration). Top row: bone marrow cells stained for expression of mouse B220 (*x*-axis) and mouse Igκ (*y*-axis). Middle row: spleen cells stained for expression of mouse B220 (*x*-axis) and mouse IgD (*y*-axis). Bottom row: peritoneal cells stained for expression of mouse B220 (*x*-axis) and mouse CD5 (*y*-axis). The animals used in the bone marrow cell experiment (top row) were: mouse #4182, 1.6-month-old female wild-type control; mouse #3669, 1.3-month-old female homozygous J_HD; and mouse #3670, 1.3-month-old female homozygous J_HD/hemizygous HC1 transgene, line 57. The animals used in the spleen and peritoneal cell experiments were: mouse #3847, 0.8-month-old female wild-type control; mouse #3672, 1.0-month-old female homozygous J_HD; and mouse #3673, 1.0-month-old female homozygous J_HD/hemizygous HC1 transgene, line 57. The relative number of cells in each of the displayed rectangular gates is given as a percent of a wide scatter gate, set to include both small, conventional, as well as larger, pre-B and B-1 cells.

horseradish peroxidase-conjugated polyclonal rabbit anti-human IgM(fc), cat. no. 309–035–095, Jackson Immuno Research, West Grove, PA]. For detection of antigen specific antibodies we coated microtiter wells with antigen and developed with peroxidase-conjugated human heavy chain isotype specific antisera. We detected bound peroxidase by incubation with hydrogen peroxide and 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid, Sigma, St Louis, MO). The reaction product is measured by absorption at 415 nm and corrected for absorption at 490 nm.

Flow cytometry

We prepared single cell suspensions from spleen, bone marrow and peritoneal cavity and lysed red cells with NH_4Cl, as described by Mishell and Shiigi (42). The lymphocytes are stained with the following reagents: phycoerythrin-conjugated anti-mouse Igκ (clone X36; Becton Dickinson, San Jose, CA), FITC-conjugated anti-mouse IgD (clone SBA 1, Southern Biotechnology Associates, Birmingham, AL), FITC-conjugated anti-mouse CD5 (clone 53-7.3; Becton Dickinson), FITC-conjugated anti-mouse Igλ (clone R26-46; Pharmingen, San Diego, CA) and Cy-Chrome-conjugated anti-mouse B220 (clone RA3–6B2; Pharmingen). We analyzed the stained cells using a FACScan flow cytometer and Lysis II software (Becton Dickinson). Most macrophages, neutrophils and residual red cells are excluded by gating on forward and side scatter.

Results

Rescue of the B cell compartment

In HC1 and HC2 transgenic animals bred into a homozygous J_HD background, serum Ig levels vary considerably between individual animals. Serum levels of human μ and human γ1 range from 1 to 100 and from 0.5 to 10 mg/ml respectively. This is 1–4 orders of magnitude below the normal serum levels of these proteins; however, individual B cells express approximately normal surface densities of Ig (16 and Figs 2 and 3) and the total number of B cells is within an order of magnitude of normal. Wild-type mice contain roughly equivalent numbers of bone marrow B cells (surface Ig^+, $B220^+$) and precursor pre-B cells (surface Ig^-, $B220^+$), while J_HD mice contain only pre-B cells in the bone marrow (28 and Fig. 2, top row). Transgenic-J_HD mice (homozygous for the J_H deletion and hemizygous for the HC1 transgene) contain both B and pre-B cells in the bone marrow, although the ratio of B cells to pre-B cells is only ~50–33% normal. This apparent retardation in the transition from pre-B to B cell may indicate that the minilocus rearranges less efficiently than the natural Ig locus. However, because we do not differentiate between immature and mature B cells within the surface $Ig^+/B220^+$ population, it also possible that the transgenic B cells are shorter lived or do not efficiently re-enter the bone marrow from the periphery. The peripheral immune tissues of the transgenic-J_HD animals also contain lower than normal levels of B cells. Approximately 60–70% of the lymphocytes in a wild-type mouse spleen are B cells ($B220^+$), while usually <50% of the lymphocytes in a transgenic-J_HD spleen are B cells (Fig. 2, middle row and Fig. 3, top row). The transgenic B cells do not express IgD because the HC1 transgene only encodes human μ and μ1. However, the lack of IgD is unlikely to be responsible for the low levels of serum Ig or the reduced numbers of B cells, because IgD^- mutant mice do not show either of these phenotypes. In fact, IgD^- mice are reported to exhibit, at most, a subtle defect in the kinetics of the immune response (43).

In the peritoneal cavity of HC1 transgenic-J_HD animals we find normal levels of $CD5^+$ B cells and ~ 25% of the normal level of conventional $CD5^-$ B cells (Fig. 2, bottom row). The transgenic peritoneal $CD5^+$ B cells are similar to the so-called B-1 cells described in normal animals (44,45): they are larger than conventional B and T lymphocytes (as measured by light scatter; data not shown), they express lower levels of B220 than the conventional B cells found in the spleen and they include a higher proportion of λ light chain expressing cells. Over 90% of the splenic B cells express κ, while up to 50% of the peritoneal B cells express λ (Fig. 3). Thus, while the level of conventional B cells is uniformly reduced in all tissues, the level of B-1 cells, which are reported to have a much greater capacity for self-renewal (46), appears to be normal in the HC1 transgenic-J_HD animals.

Transgenic B cells respond to antigen

To assess the functionality of the human Ig expressing B cells in the transgenic-J_HD animals, we immunized mice from different

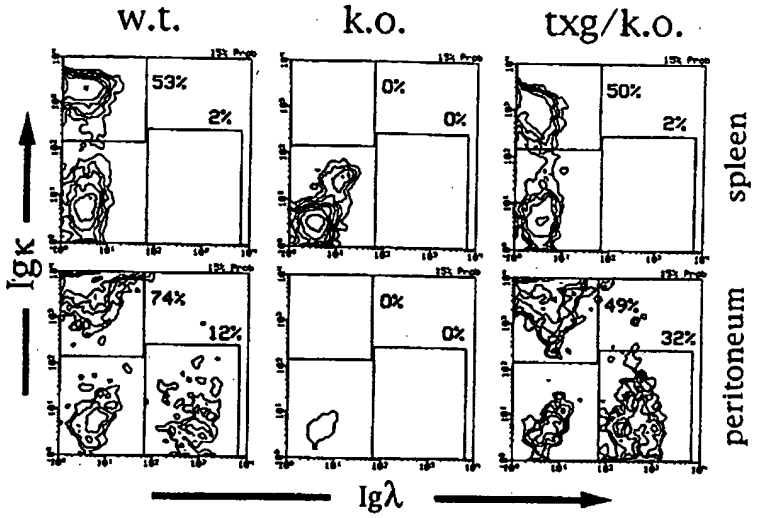

Fig. 3. The ratio of λ to κ B cells is elevated in the peritoneal cavity, relative to the spleen, in HC1 transgenic-J_HD animals. This elevation is even more pronounced than that observed in normal animals. Flow cytometric analysis of fluorescent stained cells isolated from spleen and peritoneal cavity. Left column: control animal (mouse #4182, 1.6-month-old female wild-type control). Center column: J_HD animal (mouse #3669, 1.3-month-old female homozygous J_HD). Right column: transgenic-J_HD animals (mouse #3670, 1.3-month-old female homozygous J_HD/hemizygous HC1 transgene, line 57). Top row shows spleen cells and bottom row shows peritoneal cells, stained for expression of mouse Igλ (*x*-axis) and mouse Igκ (*y*-axis). The relative number of cells in each of the displayed rectangular gates is given as a percent of a wide scatter gate, set to include both small, conventional, as well as larger, B-1 cells.

transgenic lines with a number of different antigens, including KLH, dinitrophenyl (DNP)-modified KLH and human CEA. We observe a specific human μ response to all of these antigens in the serum one week after injection with antigen and complete Freund's adjuvant. This primary response is directed against protein as well as hapten (DNP) epitopes (data not shown). Figure 4 shows a typical primary response to KLH 5 days after immunization. The animal used in this experiment contained an HC1 line 26 transgene (16). Despite the fact that the induced antibodies use only a single heavy chain V segment (V_H251), they are specific for KLH and not simply a polyreactive response to complete Freund's adjuvant.

Class switching

In normal mice, repeated exposure to antigen results in a secondary response in which reactive B cells within germinal centers are exposed to factors that promote the recombinational deletion of the μ heavy chain gene, resulting in expression of another heavy chain class such as IgG (10,11,47). This deletion event is usually mediated by non-homologous recombination between the so-called switch regions located upstream of each of the constant region genes except δ (48,49). We find that in transgenic-J_HD mice, repeated exposure to antigen results in the production of human γ1 antibodies as well as μ antibodies. We injected human CEA into transgenic-J_HD mice at weekly intervals and monitored the serum levels of antigen-specific IgM and IgG1 over a period of 4 weeks (Fig. 5). At 1 week there is a detectable IgM response but no IgG1 response. However, the IgG1 response is greater than the IgM response after 2 weeks and it continues to increase while the IgM response remains relatively constant. This pattern—an initial IgM reaction followed by an IgG reaction—is typical of a secondary immune response; and it suggests that *cis*-acting sequences included in the transgene may be responding to cytokines that direct class switching. However, because the minilocus transgenes include only a subset of the sequences contained within the natural heavy chain locus, it is

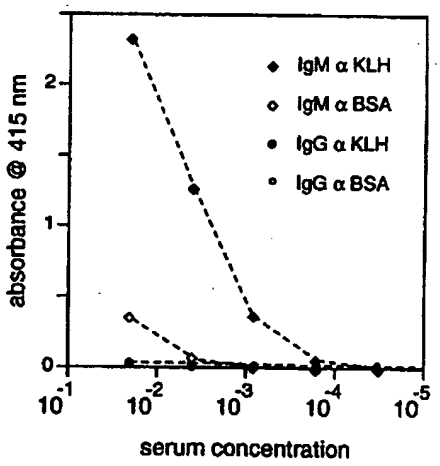

Fig. 4. Specific primary immune response from transgenic B cells. A 4-week-old female HC1 line 26 transgenic animal (#2599) in a homozygous JHD background was injected i.p. with 50 mg KLH in complete Freund's adjuvent. Serum was collected 5 days later and diluted into microtiter wells coated with KLH or BSA. The presence of specific antibody was then detected with horseradish peroxidase-linked antibodies against human IgM and human IgG.

possible that an unusual mechanism is responsible for the observed isotype shift. We have considered three possible mechanisms for expression of non-μ isotypes, each of which have been discussed in the literature. These mechanisms are: alternative splicing, which does not involve deletion of the μ gene; 'δ-type' switching, which involves deletion of the μ gene via homologous recombination between flanking repeat sequences; and non-homologous recombination between switch regions. The results of our experiments, described below, support a switch region recombination model.

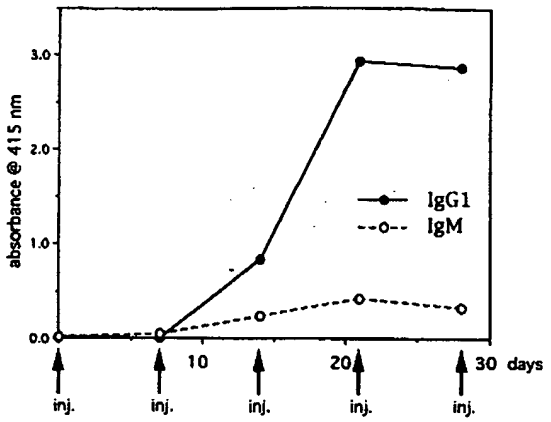

Fig. 5. Isotype specific response of transgenic animals during hyperimmunization. Relative serum levels of antigen specific antibodies—that comprise human μ or human γ1 heavy chains—during the course of a 1 month immunization schedule. The relative levels of reactive human μ and γ1 are indicated by a colorimetric ELISA assay (*y*-axis). We immunized three 7- to 10-week-old male HC1 line 57 transgenic animals (#1991, #2356 and #2357), in a homozygous J_HD background, by intraperitoneal injections of CEA in Freund's adjuvant. We gave each animal 100 mg of CEA with complete Freund's on day 0, followed by 100 mg of CEA with incomplete Freund's on days 7, 14, 21 and 28. The figure depicts binding of 250-fold dilutions of pooled serum (collected prior to each injection) to CEA coated microtiter wells. The presence of CEA specific human antibody is detected with horseradish peroxidase-linked antibodies specific for human IgM Fc and human IgG Fc.

Two types of non-deletional alternative splicing mechanisms can be invoked to explain an isotype shift. First, it is possible that a single transcript covering both μ and γ1 is expressed from the transgene; this transcript could be alternatively spliced in response to cytokines induced by exposure to antigen (50). Alternatively, a cytokine induced sterile transcript initiating upstream of γ1 could be *trans*-spliced to the μ transcript (51). If either of these mechanisms are responsible for the expression of human γ1 sequences, then we would expect to be able to isolate hybridomas that express both μ and γ1. However, although we have screened several hundred hybridomas expressing either human μ or human γ1, we have not found any such double producer (μ^+, $\gamma1^+$) hybridomas. This suggests that expression of γ1 is accompanied by deletion of the μ gene.

Deletion of the μ gene could be mediated by non-homologous recombination between the μ and γ1 switch regions or by homologous recombination between the two flanking 400 bp direct repeats ($\sigma\mu$ and $\Sigma\mu$) that are included in the HC1 and HC2 transgenes. Deletional recombination between $\sigma\mu$ and $\Sigma\mu$ appears to be responsible for the IgD^+, IgM^- phenotype of some human B cells (52,53). While the first mechanism, non-homologous switch recombination, should generate switch products of varying lengths (54), the second mechanism, $\sigma\mu/\Sigma\mu$ recombination, should always generate the same product. We performed a Southern blot analysis of genomic DNA isolated from three hybridomas (Fig. 6), one expressing μ and two expressing γ1. We find genomic rearrangements upstream of the transgene γ1 only in the two the γ1 expressing hybridomas. The resulting genomic structures differ by ~0.2 kb, as would be expected for individual recombination events involving the μ and γ1 switch regions. Furthermore, neither of the observed structures is compatible with homologous recombination between $\sigma\mu$ and $\Sigma\mu$. Our results are therefore consistent with a model for γ1 isotype expression mediated by deletional non-homologous recombination between the transgene encoded μ and γ1 switch regions.

Trans-switching

In addition to human γ1, we find mouse γ in the serum of HC1 and HC2 transgenic-J_HD mice. We (C. S. Woodhouse and S. L. O'Donnell, data not shown) have also obtained mouse γ expressing hybridomas from these animals. Because the non-transgenic homozygous J_HD animals do not express detectable levels of mouse Igs, we attribute the expression of mouse γ in the HC1 and HC2 transgenic-J_HD animals to the phenomenon of *trans*-switching described by Selsing and co-workers (23,24). All of the transgenic hybridomas that we have analyzed express either mouse or human constant region sequences, but not both. It is therefore unlikely that a *trans*-splicing mechanism (51) is involved. We used PCR amplification to isolate cDNA clones of *trans*-switch products and determined the nucleotide sequence of 10 of the resulting clones (Fig. 7). The 5′ oligonucleotide in the PCR amplification is specific for the transgene encoded V_H251 and the 3′ oligonucleotide is specific for mouse γ1, γ2b and γ3 sequences. We find examples of *trans*-switch products incorporating all three of these mouse constant regions.

Somatic mutation

Approximately 1% of the nucleotides within the variable regions of the *trans*-switch products shown in Fig. 7 are not germline encoded. This is presumably due to somatic mutation, which has been previously reported for heavy chain variable region transgenes that have recombined into the endogenous mouse heavy chain locus (24,25). Because the mutated sequence has been translocated to the endogenous locus, the *cis*-acting sequences directing these mutations could be located anywhere 3′ of the mouse γ switch. However, as we discuss below, we also observe somatic mutation in VDJ segments that have not undergone such translocations; and this result indicates that sequences required for heavy chain somatic mutation are included in the transgene.

To determine if the HC1 and HC2 constructs include sufficient *cis*-acting sequences for somatic mutation to occur in the transgenic-J_HD mice, we isolated and partially sequenced cDNA clones derived from two independent HC1 transgenic lines and one HC2 line. We find that some of the γ1 transcripts from transgenic-J_HD mice contain V regions with extensive somatic mutations. The frequency of these mutated transcripts appears to increase with repeated immunizations. Figure 8 shows two sets of cDNA sequences: one set is derived from an HC1 (line 26) transgenic-J_HD mouse that we immunized with a single injection of antigen 5 days before we isolated RNA; the second set is derived from an HC1 (line 26) transgenic-J_HD mouse that we hyperimmunized by injecting antigen on three different days beginning 5 months before we isolated RNA. Only two of the 13 V regions from the 5 day post-exposure mouse contain any non-germline encoded nucleotides. Each of these Vs contains only a single nucleotide change. In contrast, none of the 13 V sequences from the hyperimmunized animal are completely germline.

Comparison of μ and γ1 transcripts isolated from a single tissue sample shows that the frequency of somatic mutations is higher in transgene copies that have undergone a class switch. We isolated and partially sequenced 47 independent μ and γ1 cDNA clones

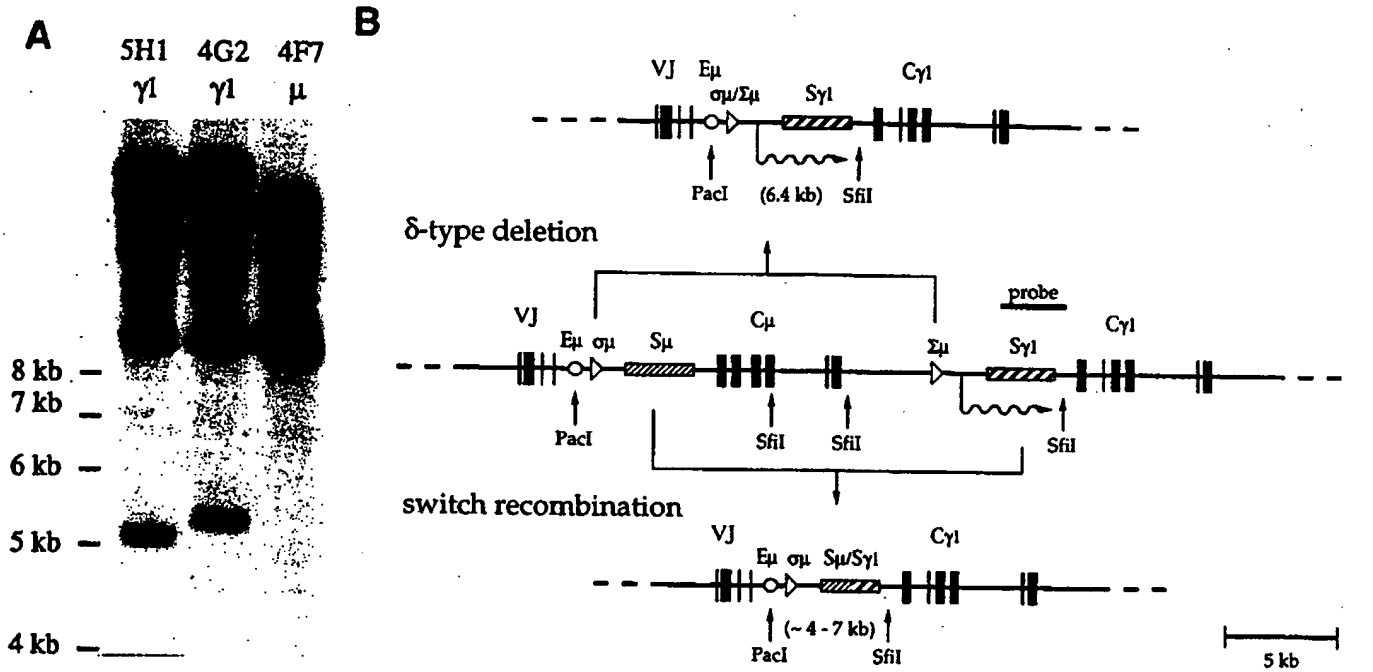

Fig. 6. Expression of transgene encoded γ1 isotype is mediated by class switch recombination. The genomic structure of integrated transgenes in two different human γ1 expressing hybridomas is consistent with recombination between the μ and γ1 switch regions. (A) Southern blot of *PacI – SfiI* digested DNA isolated from three transgene expressing hybridomas. From left to right: clone 92 – 09A-5H1 – 5, human $\gamma1^+/\mu^-$; clone 92 – 09A-4G2 – 2, human $\gamma1^+/\mu^-$; clone 92 – 09A-4F7-A5 – 2, human $\gamma1^-/\mu^+$. All three hybridomas are derived from a 7-month-old male mouse hemizygous for the HC1 – 57 integration and homozygous for the J_HD disruption (mouse #1991). The blot is hybridized with a probe derived from a 2.3 kb *BglII – SfiI* DNA fragment spanning the 3′ half of the human γ1 switch region. No switch product is found in the μ expressing hybridoma, while the two γ1 expressing hybridomas, 92 – 09A-5H1 – 5 and 92 – 09A-4G2 – 2, contain switch products resulting in *PacI – SfiI* fragments of 5.1 and 5.3 kb respectively. (B) Diagram of two possible deletional mechanisms by which a class switch from μ to γ1 can occur. The human μ gene is flanked by 400 bp direct repeats (σμ and Σμ) which can recombine to delete μ (49,50). Class switching by this mechanism will always generate a 6.4 kb *PacI – SfiI* fragment, while class switching by recombination between the μ and γ1 switch regions will generate a *PacI – SfiI* fragment between 4 and 7 kb, with size variation between individual switch events. The two γ1 expressing hybridomas examined in panel (A) appear to have undergone recombination between the μ and γ1 switch regions.

from a hyperimmunized HC1 line 57 transgenic-J_HD mouse (Fig. 9). Most of the μ cDNA clones are unmodified relative to the germline sequence, while >50% of the γ1 clones contain multiple non-germline encoded nucleotides. It thus appears that the γ1 expressing cells are distinct from the μ expressing cells and that, while the two processes are not necessarily linked, class switching and somatic mutation are taking place in the same sub-population of B cells.

Although we do not find extensive somatic mutation of the V_H251 gene in non-hyperimmunized HC1 transgenic mice, we have found considerable somatic mutation in V_H56p1 and V_H51p1 genes in a naive HC2 transgenic mouse (Fig. 10).

Discussion

The human sequence minilocus transgenes, HC1 and HC2, partially restore the B cell compartment in mice carrying disrupted endogenous heavy chain loci. The transgenic-J_HD mice have ~33% of the normal level of conventional B cells in the bone marrow, spleen and peritoneal cavity, consistent with a model in which transgene VDJ joining is less efficient than rearrangement at the natural heavy chain locus. In contrast, the level of B-1 cells in the peritoneum appears to be normal. Given the capacity for self renewal observed in normal B-1 cells (46), this population may be far less sensitive to the kinetics of B cell ontogeny than the conventional B cell population. Whatever is responsible for the reduced size of the conventional B cell population, once the transgenic cells are formed they appear to be functional. And, despite a limited repertoire—in the HC1 transgenics, the only source of primary heavy chain diversity comes from VDJ joining at CDR3—the transgenic cells respond specifically to antigen. Because the transgenic-J_HD animals still have intact mouse light chain loci, much of the specificity of the antigen response could be determined by endogenous light chains. We find it interesting that the $CD5^+$ B cell population in the peritoneum is even more enriched for λ^+ cells in the HC1 transgenic-J_HD animals than in normal animals. Almost half the B cells in the peritoneum express receptors encoded by a single V_H gene segment and, at most, three V_λ gene segments.

Class switch recombination of introduced heavy chain transgenes has been previously reported (23,24). However, because the *trans*-switch recombination events described in those experiments involved transgene encoded μ and endogenous γ switch sequences, they do not completely define the *cis*-acting sequences required for switching. While we also observe *trans*-switching in our system, the introduced heavy chain minilocus transgenes discussed in this paper include both μ and γ1 switch regions and are capable of undergoing transgene-autonomous class switch recombination.

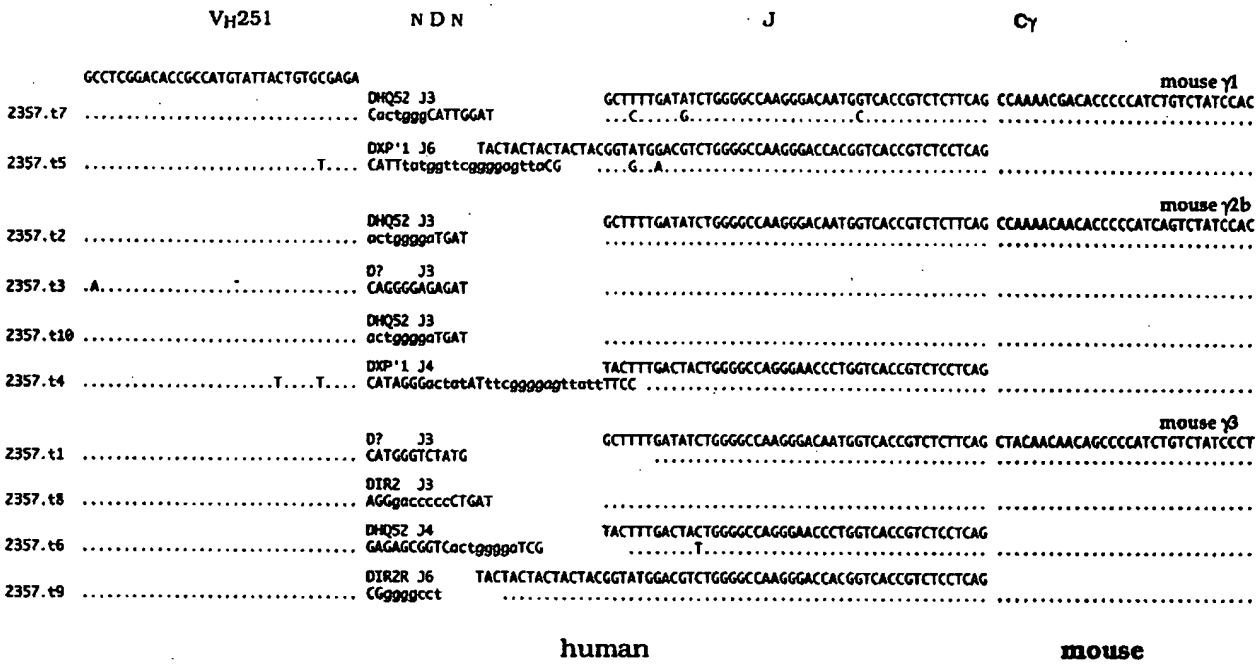

Fig. 7. Chimeric human–mouse Ig heavy chains generated by *trans*-switching. cDNA clones of *trans*-switch products were generated by reverse transcription and PCR amplification of a mixture of spleen and lymph node RNA isolated from a hyperimmunized HC1 transgenic-J_HD mouse (#2357; see legend to Fig. 5 for description of animal and immunization schedule). The partial nucleotide sequence of 10 randomly picked clones is shown. Germline sequence is shown at the top; nucleotide changes from germline are given for each clone. A period indicates identity with germline sequence, capital letters indicate no identified germline origin. The sequences are grouped according to J segment usage. The germline sequence of each of the J segments is shown. Lower case letters within CDR3 sequences indicate identity to known D segment included in the HC1 transgene. The assigned D segments are indicated above each sequence. Unassigned sequences could be derived from N region addition or somatic mutation or in some cases they are simply too short to distinguish random N nucleotides from known D segments.

Furthermore, transgene class switching appears to be taking place in the same population of B cells that are involved in the secondary response to antigen, implying that the transgene class switch is regulated by cytokines. This result has implications for identifying those *cis*-acting elements responsible for class switching, highlighting the importance of sequences located immediately upstream of the switch region. In normal mice there is abundant evidence that class switching is regulated by sequences associated with each of the individual non-μ switch regions. Individual B cells enter distinct maturation programs determined by the levels and types of cytokines and other switch factors they are exposed to and marked by, the isotype of the class switch they undergo. For example: lipopolysaccharide and IL-4 can specifically induce switching to IgG1 and subsequently IgE in mouse B cells (10,11,55,56). Furthermore, individual B cells that have undergone a class switch usually recombine identical heavy chain isotypes at both the active and inactive allele (57,58). Gu *et al.* (59) demonstrated that this isotype choice is not dependent upon the upstream μ switch region; they inactivated the μ switch by deletion of J_H and the J–μ intronic enhancer and found that although this deletion suppressed switch recombination to the mutant μ allele in hemizygous mice, the mutant γ1 allele still underwent a gene rearrangement event in IgG1$^+$ cells. The authors suggested that this event involves partial internal deletion of the γ1 switch region. The observed rearrangements might also involve *trans*-switching to the wild-type μ allele. In either case, the results suggest that the μ and γ switch regions are activated independently. After recombination, the switch product takes on the characteristics of the μ switch region and loses the characteristics of the γ switch region. Although the class-switched heavy chain locus is still an active, 'μ-like' substrate for further switching events to downstream isotypes (60–62), it does not continue to undergo further deletions and potentially destructive *trans*-switching events that would be characteristic of an active γ switch region. This transformation of the non-μ switch region is presumably caused by the deletion of switch-directing sequences located upstream of the site of switch recombination. The sterile transcripts, which initiate upstream of the switch regions and can be induced by cytokines that also specifically induce switch recombination, may be controlled by the same *cis*-acting sequences (63–67). Sequences that are closely linked to these sterile transcripts are clearly important, e.g. targeted deletion of the first exon of the mouse γ1 or γ2b sterile transcript, together with upstream flanking sequences, eliminates class switching to γ1 and γ2b respectively (68,69). In addition, replacement of the ϵ IL-4-responsive germline promoter with a constitutive promoter causes IL-4 independent class switching to IgE *in vitro* (70). These results establish that upstream *cis*-acting sequences define the functionality of the individual switch regions and are necessary for class switching. Our observation—that class switching within the HC1 transgene is largely confined to cells involved in secondary responses and does not occur randomly across the entire B cell population—suggests that the minimal sequences contained within the transgene are sufficient. Because the γ sequences included in this construct begin only 116 nucleotides upstream of the start site of the γ1 sterile transcript (38), the switch regulatory region appears to be compact. Further experiments are required to determine if the observed regulation

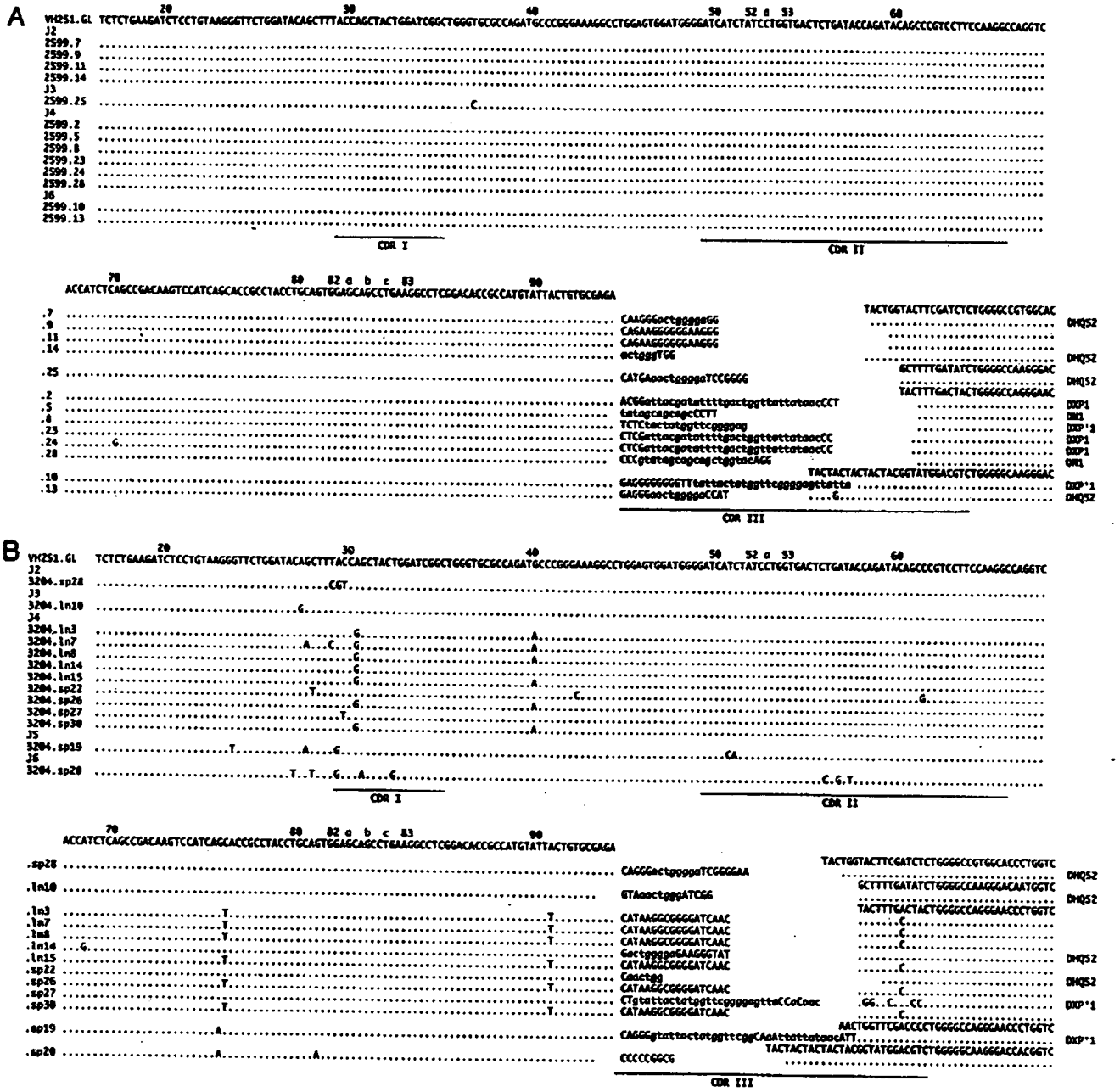

Fig. 8. The rearranged V_H251 transgene undergoes somatic mutation in a hyperimmunized mouse. The partial nucleotide sequence of IgG heavy chain variable region cDNA clones from HC1 line 26 mice exhibiting (A) primary and (B) secondary responses to antigen. See legend to Fig. 7 for explanation of symbols. (A) Primary response: 13 randomly picked $V_H251-\gamma1$ cDNA clones. A 4-week-old female HC1 line 26-J_HD mouse (#2599) was given a single injection of KLH and complete Freund's adjuvant; spleen cell RNA was isolated 5 days later. The serum response for this animal is shown in Fig. 4. (B) Secondary response: 13 randomly picked $V_H251-\gamma1$ cDNA clones. A 2-month-old female HC1 line 26-J_HD mouse (#3204) was given three injections of HEL and Freund's adjuvant over 1 month (a primary injection with complete adjuvant and boosts with incomplete at 1 and 3 weeks); spleen and lymph node cell RNA was isolated 4 months later. Lymph node and spleen derived sequences are marked 'ln' and 'sp' respectively.

is correct in detail. However, structural comparisons of mouse and human γ genes (71), as well as differences in IgG subclass responses (72), suggest that the individual subclasses evolved independently in the two lineages. It is therefore surprising that a human $\gamma1$ switch region is regulated at all in mouse cells *in vivo*.

As with class switching, most previous studies of somatic mutation in heavy chain transgenes involved translocation to the endogenous heavy chain locus (24,25,73) and could not therefore define the required *cis*-acting sequences. The single reported observation of somatic mutation events in non-translocated transgenes found a very low frequency of non-germline encoded nucleotides. Sohn *et al.* (73) looked at 16 IgM expressing hybridomas from an immunized

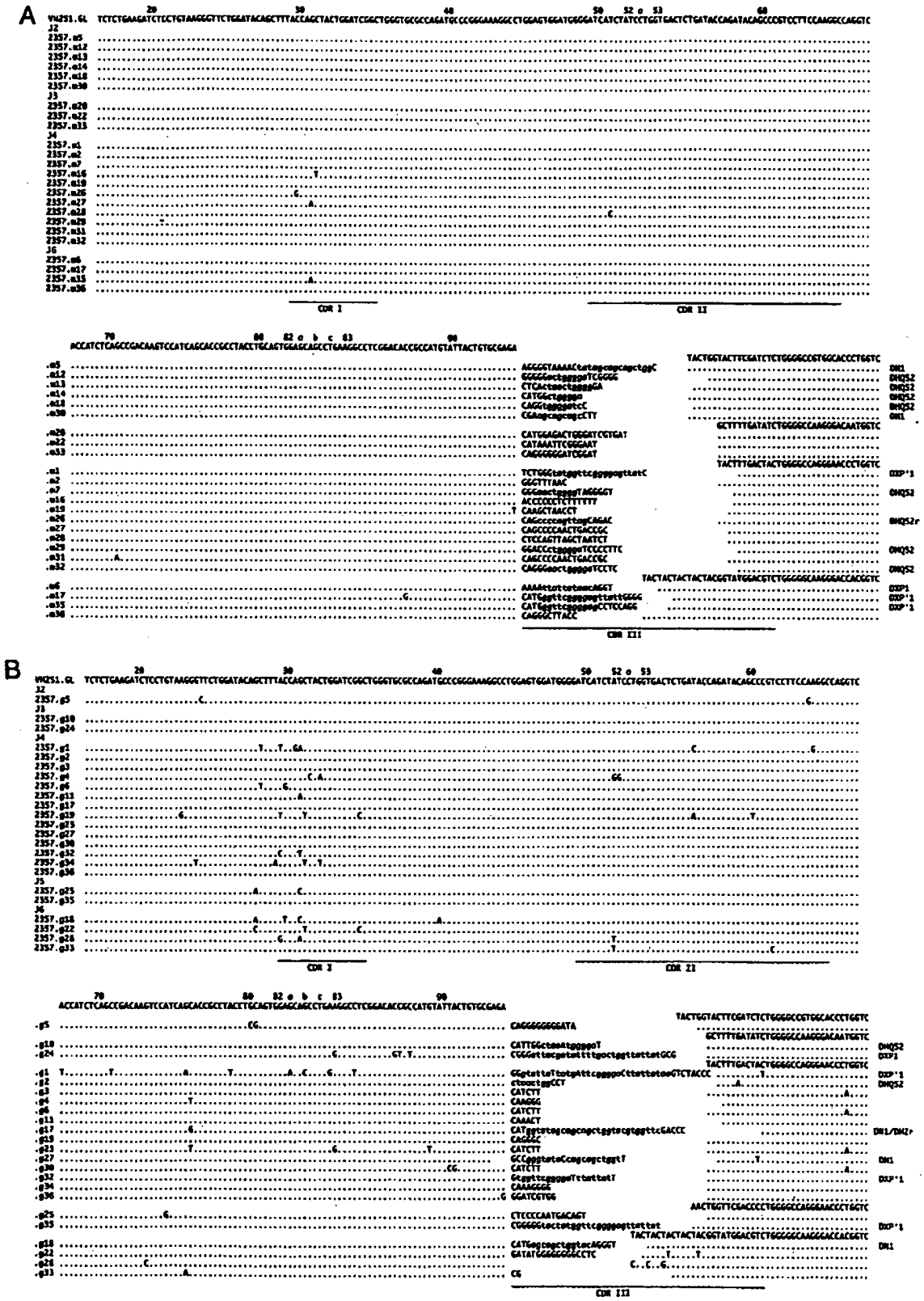

Fig. 9. Extensive somatic mutation is confined to γ1 sequences: somatic mutation and class switching occur within the same population of B cells. Partial nucleotide sequence of V_H251 cDNA clones isolated from spleen and lymph node cells of HC1 line 57 transgenic-J_HD mouse (#2357) hyperimmunized against CEA (see Fig. 5 for immunization schedule). (A) IgM: 24 randomly picked $V_H251-\mu$ cDNA clones. (B) IgG: 23 randomly picked $V_H251-\gamma1$ cDNA clones. See legend to Fig. 7 for explanation of symbols.

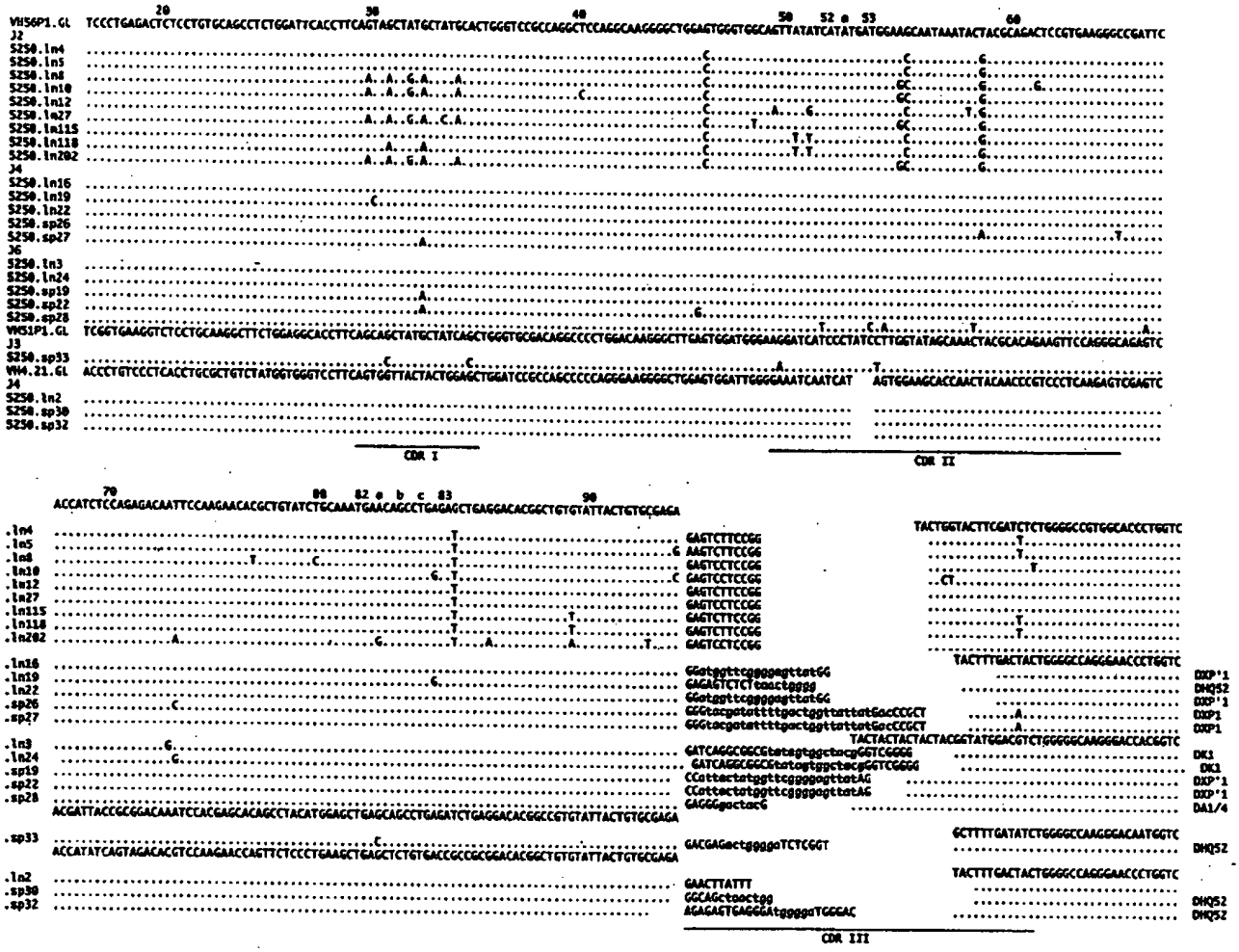

Fig. 10. V_H51P1 and V_H56P1 show extensive somatic mutation in an un-immunized mouse. The partial nucleotide sequence of IgG heavy chain variable region cDNA clones from a 9-week-old, un-immunized, female HC2 line 2550 transgenic-J_HD mouse (#5250). See legend to Fig. 7 for explanation of symbols.

mouse containing a μ transgene. Two of the 16 hybridomas appeared to express mutated transcripts by RNase protection assay and nucleotide sequence analysis confirmed that a fraction of the transcripts from one of the hybridomas contained two non-germline encoded nucleotides and some of the transcripts from the other contained four base changes. *Trans*-switched IgG hybridomas from the same study contained transcripts with an order of magnitude higher level of somatic mutation. Furthermore, in hybridomas expressing both IgM and IgG, the γ transcripts were highly mutated while the μ transcripts contained mutations at a level only 3-to 4-fold above the observed background of PCR-introduced errors. The authors concluded that while non-translocated μ transgenes could undergo somatic mutation, important *cis*-acting elements for the high level somatic mutation found in affinity maturation must be located downstream of non-μ switch sequences. Furthermore, these sequences must act locally, as γ transcripts show higher levels of mutation than μ transcripts synthesized from within the same transgene cluster in double producer hybridomas. Our results demonstrate that these important *cis*-acting regulatory elements are either closely linked to individual γ genes or associated with the 3′ heavy chain enhancer included in the HC1 and HC2 transgenes.

Experiments with light chain transgenes have demonstrated that sequences required for somatic mutation of the κ locus are closely associated with the coding exons and downstream sequences are important for high levels of mutation (22). Because the HC1 and HC2 heavy chain inserts undergo transgene-autonomous class switching—which can serve as a marker for sequences that are likely to have been somatically mutated—we were able to easily find hypermutated transcripts that did not originate from translocations to the endogenous locus. We found somatically mutated γ transcripts in three out of three independent transgenic lines that we tested (two HC1 lines and one HC2 line). It is therefore unlikely that sequences flanking the integration sites of the transgene affect this process; instead, the transgene sequences are sufficient to direct somatic mutation. It may be possible to use this system to further map these *cis*-acting elements by constructing new transgenes consisting of subsets of the sequences in the HC1 minilocus.

In conclusion, our results demonstrate that a human sequence minilocus can complement the endogenous mouse heavy chain locus, thus rescuing B cell development and function in an otherwise B cell deficient mutant mouse strain. The HC1 and HC2 miniloci

differ from the intact mouse heavy chain locus in several respects: upon integration, the miniloci are isolated from any unidentified *cis*-acting regulatory sequences that are not closely linked to the coding sequences included in the transgene; the miniloci are also potentially affected by novel *cis*-acting regulatory sequences associated with the integration site of the transgene; the miniloci encode far less combinatorial diversity than the authentic locus; and, finally, the miniloci encode a human protein that must associate with mouse proteins to form a functional B cell receptor complex. Despite these differences, we find that the transgenes are able to complement the J_HD mutation. Therefore, the proximal regulatory sequences included in the transgene are sufficient for rearrangement and expression of a primary repertoire and for directing heavy chain somatic mutation and class switching. These sequences are all closely linked to protein coding segments in the natural IgH locus; the rat 3′ enhancer, which is normally located 25 kb downstream of C_α, is the most distant. Furthermore, the resulting human proteins can replace the mouse heavy chain in B cell development and, despite a limited primary repertoire, can participate in immune responses to a variety of antigens.

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Abbreviations

CEA	**carcinoembryonic antigen**
CDR	**complementarity determining region**
D	diversity
DNP	dinitrophenyl
J_H	heavy chain joining
J_HD	heavy chain joining segment deletion
KLH	keyhole limpet hemocyanin
PCR	polymerase chain reaction
V	variable

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